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A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein

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Herpes simplex virus type 1 immediate-early protein Vmw110 is a non-specific activator of gene expression and is required for efficient initiation of the viral lytic cycle. Since Vmw110-deficient viruses reactivate inefficiently in mouse latency models it has been suggested that Vmw110 plays a role in the balance between the latent and lytic states of the virus. The mechanisms by which Vmw110 achieves these functions are poorly understood. Vmw110 migrates to discrete nuclear structures (ND10) which contain the cellular PML protein, and in consequence PML and other constituent proteins are dispersed. In addition, Vmw110 binds to a cellular protein of ~135 kDa, and its interactions with the 135 kDa protein and ND10 contribute to its ability to stimulate gene expression and viral lytic growth. In this report we identify the 135 kDa protein as a novel member of the ubiquitin-specific protease family. The protease is distributed in the nucleus in a micropunctate pattern with a limited number of larger discrete foci, some of which co-localize with PML in ND10. At early times of virus infection, the presence of Vmw110 increases the proportion of ND10 which contain the ubiquitin-specific protease. These results identify a novel, transitory component of ND10 and implicate a previously uncharacterized ubiquitin-dependent pathway in the control of viral gene expression.

Keywords: herpesvirus/ICP0/PML nuclear domain/ ubiquitin-specific protease/Vmw110

Introduction

Herpes simplex virus type 1 (HSV-1) is a common human pathogen which attains a life-long latent state in sensory neurones after initial infection at the periphery. The establishment of latency and the subsequent episodes of reactivation are fundamental to the clinical importance of herpes simplex viruses and undoubtedly contribute substantially to their evolutionary success, as latency allows the virus to evade the immune system. The pattern of viral gene expression during lytic infection, when at least 76 genes are expressed from the 152 kb genome (McGeoch *et al.*, 1993 and references therein), contrasts with that of latency when only one active viral transcription unit of unknown function has been detected (for a review see Fraser *et al.*, 1992). HSV-1 genes can be divided into Immediate-Early (IE), Early and Late temporal classes depending on their time-course of synthesis and requirements for prior viral gene expression and DNA replication (reviewed by Roizman and Sears, 1990). Transcription of the IE genes is stimulated by the viral tegument protein VP16, which interacts with Oct-1 and other cellular proteins (Wilson *et al.*, 1993 and references therein), and in turn four of the five IE gene products have roles in the normal expression of later classes of viral genes. The product of IE gene 3 is the major viral transcriptional transactivator Vmw175 (also known as ICP4), which forms a tripartite complex with TFIID and TFIIB to stimulate transcription from early and late promoters (Smith *et al.*, 1993). IE gene 2 encodes Vmw63 (ICP27), which most likely acts at the post-transcriptional level to influence fully efficient expression of late genes (McCarthy *et al.*, 1989; Sandri-Goldin and Mendoza, 1992; Phelan *et al.*, 1993). Vmw68 (ICP22), the product of IE gene 4, is required for normal late gene expression in some cell lines and its presence results in the appearance of an underphosphorylated form of RNA polymerase II (Poffenberger *et al.*, 1993; Rice *et al.*, 1995). The product of IE gene 1 is the RING finger protein Vmw110 (ICP0), a strong and promiscuous activator of gene expression in transfection assays which can act synergistically with Vmw175 (reviewed by Everett *et al.*, 1991).

Several lines of evidence indicate that Vmw110 might have a specific role in influencing the latent–lytic switch. Viruses with lesions which inactivate Vmw110 can be propagated in culture, but they exhibit a marked multiplicity-dependence for the onset of lytic infection such that their probability of initiating a productive infection is much reduced (Stow and Stow, 1986; Sacks and Schaffer, 1987). The Vmw110-mutant virus particles which fail to begin replication enter a quiescent state from which they can be reactivated by superinfection (Stow and Stow, 1989). Tissue culture systems have been developed in which HSV-1 genomes can be established in a quiescent or latent state, and their reactivation can be induced by provision of exogenous Vmw110 (Harris *et al.*, 1989; Zhu *et al.*, 1990). Furthermore, Vmw110-deficient viruses reactivate inefficiently in mouse latency models (Clements and Stow, 1989; Lieb *et al.*, 1989; Cai *et al.*, 1993). Accordingly, it has been suggested that Vmw110 plays a role in the balance between the latent and lytic states; in its presence, the latter is favoured.

The mechanisms by which Vmw110 achieves these functions are poorly understood. It is likely that cell factors also play major roles in controlling the replication status of the virus. For example, the multiplicity-dependent defect of Vmw110-deficient viruses can be modulated by both cell type and cell cycle status (Cai and Schaffer, 1991; Yao and Schaffer, 1995). Therefore, interactions

between the virus and the cell, and in particular between Vmw110 and cellular proteins, are likely to be highly important for the biology of the virus. Two such interactions have been described; first, that between Vmw110 and the PML-containing nuclear bodies, and secondly, the direct binding of Vmw110 to a cellular protein of ~135 kDa.

Nuclear bodies (also known as PODs and referred to herein as ND10) are punctate structures associated with the nuclear matrix which have been shown to contain at least four different proteins (Ascoli and Maul, 1991; Stuurman *et al.*, 1992; Dyck *et al.*, 1994; Korioth *et al.*, 1995). One of these proteins is PML, a RING finger protein which becomes fused to the retinoic acid receptor alpha (RARα) as a result of the t(15;17) chromosomal translocation in promyelocytic leukaemic blasts (de The *et al.*, 1991; Goddard *et al.*, 1991; Kakizuka *et al.*, 1991; Kastner *et al.*, 1992; Pandolfi *et al.*, 1992). A consequence of this translocation is the disruption of ND10 from their normal appearance of an average of ~10 punctate foci per nucleus, such that multiple small nuclear and cytoplasmic speckles are formed (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). Since treatment with retinoic acid restores the normal ND10 staining pattern and results in differentiation of the blasts, ND10 have been suggested to play a role in the regulation of cell growth and proliferation. Interestingly, recent work has shown that ND10 are modified during infection by a number of DNA viruses (Maul *et al.*, 1993; Kelly *et al.*, 1995; Doucas *et al.*, 1996). In the case of HSV-1, Vmw110 migrates to ND10 in the early stages of infection, and in consequence PML and other constituent proteins are dispersed (Everett and Maul, 1994; Maul and Everett, 1994). It is intriguing that mutations in Vmw110 that affect its roles in gene expression and the initiation of viral infection also alter the interaction of Vmw110 with ND10, thus implying that ND10 are involved in the onset of HSV-1 gene expression and infection. Indeed, it has been recently shown that ND10 are preferred locations within the nucleus where both HSV-1 and adenovirus DNA replication begin (Ishov and Maul, 1996; Maul *et al.*, 1996).

To investigate the molecular basis of the functions of Vmw110, we searched for cellular proteins to which it might bind and we found a strong and specific interaction between Vmw110 and a cellular protein of ~135 kDa (Meredith *et al.*, 1994, 1995). Deletion of the region of Vmw110 which binds to the 135 kDa protein reduces significantly its ability to stimulate gene expression and viral lytic growth (Everett, 1988; Meredith *et al.*, 1995), and a deletion which overlaps this region eliminates the ability of Vmw110 to reactivate latent virus in an *in vitro* latency system (Zhu *et al.*, 1990). In this report we identify the Vmw110-associated 135 kDa protein as a novel member of the ubiquitin-specific protease family which can be found associated with a subset of ND10. The expression of Vmw110 at early times of virus infection results in the presence of the protease in a higher proportion of ND10. These results define a novel transitory component of ND10 and implicate a previously uncharacterized ubiquitin-dependent pathway in both the function of ND10 and the control of viral gene expression.

Results

Cloning of a cDNA encoding a cellular Vmw110-associated protein

We have shown that glutathione *S*-transferase (GST) fusion proteins containing the C-terminal 180 residues of Vmw110 bind to a cellular protein of ~135 kDa in unfractionated cell extracts, and that a protein of identical gel mobility co-immunoprecipitates with Vmw110 from virus-infected cells (Meredith *et al.*, 1994, 1995). In both assays, residues 594–633 of Vmw110 were found to be essential for this interaction, and because these residues also contribute to the functional and biological properties of Vmw110 (Everett, 1988; Meredith *et al.*, 1995) it was considered of interest to identify the interacting cellular protein. The 135 kDa protein was purified by large-scale GST pull-down experiments using a GST fusion protein including residues 594–775 of Vmw110, and six internal peptide sequences were obtained (see Figure 1). An oligonucleotide based on peptide 45 was used to screen a HeLa cDNA library, and a family of overlapping cDNA clones was isolated. Sequence analysis revealed an open reading frame of 1102 codons, encoding a protein of predicted molecular weight 128 kDa, which included close or exact matches to all six of the original peptide sequences (Figure 1).

A number of independent cDNA clones were obtained such that the majority of the predicted coding sequence was confirmed in at least two clones. The extreme 3′ end sequences were identified by a match of peptide 39 with an entry in the NCBI dbest database, and an RT–PCR clone was isolated which linked this sequence to the cloned cDNAs. The true 5′ end of the mRNA remains to be identified, but the presumed initiating methionine is likely to be correct as there are no further ATG triplets in over 200 bp of sequence further 5′ that is available. Additionally, this presumed 5′ untranslated leader region is exceptionally GC-rich (an average of 90%) which indicates that it is highly unlikely to be protein-coding. Three independent cDNA clones spanning at least part of this leader were isolated, which proves that these unusual sequences are not a cloning artefact.

Analysis of the cDNA sequence

Comparison of the protein sequence with the NCBI nr database showed that the Vmw110-associated protein contained excellent matches to the two conserved motifs of the ubiquitin-specific protease (USP) family (Baker *et al.*, 1992; Papa and Hochstrasser, 1993; Figure 2) and accordingly we have renamed the protein HAUSP (herpesvirus-associated ubiquitin-specific protease). Sequences outside these motifs were not highly related to other USP enzymes of mammalian origin, but HAUSP has significant similarity throughout its sequence to uncharacterized proteins encoded by *Saccharomyces cerevisiae* (SC9952X6; 59% similarity, 34% identity) and *Schizosaccharomyces pombe* (Q09879; 57% similarity, 35% identity, where data are available) (Figure 2 and data not shown). This implies that HAUSP has homologues widely conserved through eukaryotic evolution.

Apart from the USP active site domains, database comparisons did not detect any other highly conserved features within the HAUSP sequence. However, of interest

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1     MNHQQQQQQQKAGEQQLSEPEDMEMEAGDTDDPPRITQNPVINGNVALSD

51    GHNTAEEDMEDDTSWRSEATFQFTVERFSRLSESVLSPPCFVRNLPWKIM

                                                peptide 40 AF
101   VMPRFYPDRPHQKSVGFFLQCNAESDSTSWSCHAQAVLKIINYRDDEKSF

      DELIXHLFNVK                peptide 45 KVTFEVFVQADAP
151   SRRISHLFFHKENDWGFSNFMAWSEVTDPEKGFIDDDKVTFEVFVQADAP

      IGVAPDQK
201   HGVAWDSKKHTGYVGLKNQGATCYMNSLLQTLFFTNQLRKAVYMMPTEGD
                  GL N GnTCOMNSOLQcLO
                  <-USP Cys domain ->

251   DSSKSVPLALQRVFYELQHSDKPVGTKKLTKSFGWETLDSFMQHDVQELC

301   RVLLDNVENKMKGTCVEGTIPKLFRGKMVSYIQCKEVDYRSDRREDYYDI

peptide 52/2 NIFEXFVDYVAVEQLDGDNK
351   QLSIKGKKNIFESFVDYVAVEQLDGDNKYDAGEHGLQEAEKGVKFLTLPP

            peptide 52 INDRFEFPXQLPLDEFLQK
401   VLHLQLMRFMYDPQTDQNIKINDRFEFPEQLPLDEFLQKTDPKDPANYIL
                                                  Y L
                                                  <--

451   HAVLVHSGDNHGGHYVVYLNPKGDGKWCKFDDDVVSRCTKEEAIEHNYGG
        V  H G    GHY  O        W  ODD
      ---------- USP His domain-------------------------

501   HDDDLSVRHCTNAYMLVYIRESKLSEVLQAVTDHDIPQQLVERLQEEKRI
                  AYOL Y
      ----------------->

551   EAQKRKERQEAHLYMQVQIVAEDQFCGHQGNDMYDEEKVKYTVFKVLKNS

601   SLAEFVQSLSQTMGFPQDQIRLWPMQARSNGTKRPAMLDNEADGNKTMIE

651   LSDNENPWTIFLETVDPELAASGATLPKFDKDHDVMLFLKMYDPKTRSLN

701   YCGHIYTPISCKIRDLLPVMCDRAGFIQDTSLILYEEVKPNLTERIQDYD

751   VSLDKALDELMDGDIIVFQKDDPENDNSELPTAKEYFRDLYHRVDVIFCD

801   KTIPNDPGFVVTLSNRMNYFQVAKTVAQRLNTDPMLLQFFKSQGYRDGPG

                  peptide 23 LYYQQLK
851   NPLRHNYEGTLRDLLQFFKPRQPKKLYYQQLKMKITDFENRRSFKCIWLN

901   SQFREEEITLYPDKHGCVRDLLEECKKAVELGEKASGKLRLLEIVSYKII

951   GVHQEDELLECLSPATSRTFRIEEIPLDQVDIDKENEMLVTVAHFHKEVF

1001  GTFGIPFLLRIHQGEHFREVMKRIQSLLDIQEKEFEKFKFAIVMTGRHQY

      peptide 39 DFEPTPGNMSXPXPXLGLDXFNK
1051  INEDEYEVNLKDFEPQPGNMSHPRPWLGLDHFNKAPKRSRYTYLEKAIKI

1101  HN*
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Fig. 1. Predicted amino acid sequence of HAUSP. The positions of the six peptide sequences derived from the HAUSP protein sample are marked; the discrepancies between the experimental peptide sequence data and the translated cDNA sequences are probably due to difficulties in sequencing the small amount of material that was available. The highly conserved residues within the cysteine and histidine motifs of the USP family of proteins are indicated, with O depicting conserved hydrophobic residues.

is a polyglutamine tract near the N-terminus, and several regions of high predicted α helix content, including the N-terminal 19 residues and segments between residues 515 and 572, 920 and 960, and 1008 and 1043. The first three of these regions display low-level similarity to the helical bundles of the involucrin family of proteins, which form an extended flexible rod and are thought to allow multiple intermolecular interactions (Yaffe *et al.*, 1992). It is possible that the helical regions in HAUSP are involved in protein–protein binding events which influence the target specificity of the enzyme.

Analysis of the HAUSP transcript

To analyse the abundance of the HAUSP transcript, Northern blotting was performed using probes containing a 1.3 kb fragment at the 5′ end of the coding region and a 2 kb fragment further 3′. Both gave the same results, detecting two transcripts, one of ~4.5 kb and a slightly larger (perhaps alternatively spliced) band of lesser abundance (Figure 3). These transcripts were only detectable in poly(A)$^+$ RNA preparations and their low abundance (estimated by phosphorimager analysis of the blots as being at most 1% of that of γ-actin) emphasizes the specificity of the Vmw110–HAUSP interaction. Screening of the NCBI dbest database of expressed sequence tags revealed entries with precise matches in cDNA libraries derived from brain, liver, placenta, lung and melanocyte human cells. This suggests that HAUSP is expressed in a wide variety of cell types.

HAUSP is enzymatically active on model substrates

The ability of HAUSP to cleave model ubiquitin fusion protein substrates was investigated by co-expression in *Escherichia coli*. Complete coding region clones for HAUSP were constructed in T7-driven vectors in both pBR322 and pACYC184-based replicons (see Materials and methods), and these plasmids were introduced into bacteria harbouring model substrate expression cassettes in compatible replicons. These bacteria expressed a full-length protein of identical gel mobility to that of HeLa cell HAUSP both before induction and in increased amounts after induction of expression with IPTG (data not shown). As a control, plasmids expressing the yeast ubiquitin-specific protease UBP2 were analysed in parallel. A model substrate comprising the natural human Ub52 fusion protein precursor linked to GST, such that the ubiquitin sequences comprise the middle portion of the hybrid protein, was cleaved efficiently by UBP2 to yield a product of the expected size. Expression of HAUSP resulted in the same cleavage product, albeit at a reduced efficiency (Figure 4A). Similarly, both UBP2 and HAUSP cleaved the Ub-Met-β-galactosidase model substrate, although HAUSP was again less active (Figure 4B). It is not known whether the reduced activity of HAUSP in these assays is a consequence of relative expression levels, or whether it is a reflection of substrate specificity.

Analysis of the Vmw110–HAUSP interaction

Because the HAUSP cDNAs had been cloned on the basis of peptide sequences derived from a GST pull-down experiment using a segment of Vmw110, it was important to prove that the cloned cDNA encodes the protein which binds to Vmw110 in virus-infected cells. A portion of the HAUSP open reading frame was expressed in *E.coli* as a GST fusion protein and used to produce an anti-HAUSP rabbit antiserum (r29). This serum detected a band of the correct gel mobility in Western blots of HeLa cell extracts, and in addition two other bands which were also detected by the corresponding pre-immune serum (data not shown). Extracts of virus-infected cells were prepared and incubated with anti-Vmw110 Mab 11060 to immunoprecipitate Vmw110 and associated proteins. As previously reported, Vmw110 was immunoprecipitated with a cellular radiolabelled band of ~135 kDa from wild-type virus-infected extracts, but the cellular protein was undetectable when mutant viruses D12 and E52X were used (Figure 5B). Viruses D12 and E52X have deletions which remove Vmw110 codons 594–633 and 594–775 respectively, thus

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   HAUSP  Human                SKKHTGYVG LKNQGATCYM NSLLQTLFFT NQLRKAVYMM PTEGDDSSKS VPLALQRVFY
 Sc9952x6 S.cerevisiae unknown SKKVTGYVG FRNQGATCYL NSLLQSYFFT KYFRKLVYEI PTEHESPNNS VPLALQRAFY
   Q09879 S.pombe unknown      SKIATGYVG LKNQGATCYM NSLLQSLYII HAFRRIVYQI PTDSPQGKDS IAYALQRCFY
   P38187 S.cerevisiae UBPB    GDGSNKVFG YENFGNTCYC NSVLQCLYNL SSLRENILQF PKKSRESDHP RKKEMRGKKP
   P39967 S.cerevisiae UBPE    GDGSNKVFG YENFGNTCYC NSVLQCLYNI PEFRCNVLRY PERVAAVNRI RKSDLKGSKI
   P34547 C.elegans UBPX       NNANEHYYG L..FGNTCYC NSVIQALFFC RPFREKVLNY KQTLKKSGAS KDNLVTCLAD
   A49132 D.melanogaster faf   ARPTKGFCG LKNAGATCYM NSVLQQLYMV PAVRVGILRA HGAATTDGED FSGDSDLTGG
   Z54218 C.elegans unknown    RGRQFDKVG MKNDGGTCYM NAMIQQLVHV PGLSRELIAL QNIDPQLRWG DNTAALLCEL
 Sc9959x5 S.cerevisiae unknown MERRDGLSG LINMGSTCFM SSILQCLIHN PYFIRHSMSQ IHSNNCKVRS PDKCFSCALD
   P40453 S.cerevisiae UBPI     VYVSLSITG LRNLGNTCYI NSMIQCLFAA KTFRTLFISS KYKSYLQPIR SNGSHYSPKL
   P36026 S.cerevisiae UBPK     LLSELSITG LQNPCNTCYI NSIIQCLFGT TLFRDLFLTK KYRLFLNTNK YPKEVQLSRS
   P32571 S.cerevisiae doa4     NYDLDFAVG LENLGNSCYM NCIIQCILGT HELTQIFLDD SYAKHININS KLGSKGILAK
   P39944 S.cerevisiae UBP5     VLDLDLIVG LENIGNCCYM NCILQCLVGT HDLVRMFLDN TYLNFINFDS SRGSKGLLAK
   P35123 Mouse tre2 homology   PHIQPGLCG LGNLGNTCFM NSALQCLSNT APLTEYFLKD EYEAEINRDN PLGMKGEIAE
   U20657 Human unph proto-onco SHIQPGLCG LGNLGNTCFM NSALQCLSNT HPLTDYFLKD EYEAEINRDN PLGMKGEIAE
   P35125 Human tre2 proto-onco VPTEKGATG LSNLGNTCFM NSSIQCVSNT QPLTQYFISG RHLYELNRTN PIGMKGHMAK
   P39538 S.cerevisiae UBPJ     LEPASGTTG LVNLGNTCYM NSALQCLVHI PQLRDYFLYD GYEDEINEEN PLGYHGYVAR
   P40818 Human UBPX           GGSGPALTG LRNLGNTCYM NSILQCLCNA PHLADYFNRN CYQDDINRSN LLGHKGEVAE
   P25037 S.cerevisiae UBP1     LKRGGFIAG LVNDGNTCFM NSVLQSLASS RELMEFLDNN VIRTYEEIEQ NEHNEEGNGQ
   Q01476 S.cerevisiae UBP2     LPPENWPTG INNIGNTCYL NSLLQYYFSI APLRRYVLEY QKTVENFNDH LSNSGHIRRI
 Z47811x3 C.elegans unknown     VRNPNFSTG LYNSGNTCWL NCLSQVLYSI PKFRSILYHC APLSWHEQPI TNVKIENQQH
consensus                             G L N GnTCOM NSOLQcLO     O   OO
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   HAUSP  PAN YILHAVLVHS G.DNHGGHYV VYLNPKGDG. .......KWC KFDDDVVSRC TKEEAIEHNY GGHDDD.... ..........  LSVR.HCTNA YMLVYIRESK
 Sc9952x6 PYV YNLHGVLVHS G.DISTGHYY TLIKPGVED. .......QWY RFDDERVWRV TKKQVFQENF GCDRLPDEKV RTMTRGEYQN  YIIQ.RHTSA YMLVYIRQEQ
   Q09879 NCE YVLYGVLVHS G.DLHNGHYY ALLKTEKDG. .......PWY KYDDTRVTRA TLREVLEENY GGDYIMHPPF RSP.......  VKLK.RFMSA YMLLYLRKDK
   P38187 CQK YELAGIVVHM GGGPQHGHYV SLCKHEKF.. .......GWL LFDDETVEAV KEETVLEFT. ..........  ..........  .GESPNMATA YVCFIKRCIQ
   P39967 YKK YELSGVVIHM GSGPQHGHYV CICRNEKF.. .......GWL LYDDETVESI KEETVLQFT. ..........  ..........  .GHPGDQTTA YVLFYKETQA
   P34547 DRM YDLVATVVHC GATPNRGHYI TLVKSNSF.. ........WL VFDDDIVEKL EVSSMEEFSG MSTDANIQMP PGNQS.....  APQKEKRICL YSVLSGKRLR
   A49132 TTK YELTGIVVHS G.QASGGHYF SYILSKNPAN ....GKCQWY KFDDGEVTEC KMHEDEEMKA ECFGGEYMGE TYDNNLKRMQ  YRRQKRWWNA YMLFYTRCDQ
   Z54218 PLI YELVGVLAHS G.IATAGHYY SFIKERREEF RDSPHYNKWH HINDMIVSPM SFNNIEDLWY GG........ TFTQEGVFIG  LDERVRHWNA YVLFYEKKRD
 Sc9959x5 DII YELIGIVSHK G.TVNEGHYI AFCKIS.... .....GGQWF KFNDSMVSSI S.......... ..........  ..........  .QEEVLKEQA YLLFYTIRQV
   P40453 TMK YKLFGVVNHT G.TLISGHYT SLVNKDLEH. NVNIGRSKWY YFDDEVVKAD RKHGSD.... ..........  ..........  KNLKISSSDV YVLFYERVYD
   P36026 VIR YKLYGTVNHS G.NLINGHYT SVVNKEKSH. EIGLNRQVWV TFDDDYIQQH RKDRNNFEAG ..........  ..........  .KTEMSSDEV YVLFYERMDE
   P32571 PFK YELYGVACHF G.TLYGGHYT AYVKKGL... .....KKGWL YFDDTKYKPV KN........ ..........  ..........  .KADAINSNA YVLFYHRVYG
   P39944 PFR YRLYGVACHS G.SLYGGHYT SYVYKGP... .....KKGWY FFDDSLYRPI TF........ ..........  ..........  .STEFITPSA YVLFYERIF.
   P35123 PYV YDLIAVSNHY G.AMGVGHYT AYAKNRL... .....NGKWY YFDDSSVSLA S.......... ..........  ..........  .EDQIVTKAA YVLFYQRRDD
   U20657 PYV YDLIAVSNHY G.AMGVGHYT AYAKNKL... .....NGKWY YFDDSNVSWP LRIR...... ..........  ..........  .......... ..........
   P35125 KPI YNLYAISCHS G.ILSGGHYI TYAKNP.... .....NCKWY CYNDSSCEEL H.......... ..........  ..........  .PDEIDTDSA YILFYEQQGI
   P39538 GLI YDLYAVDNHY G.GLGGGHYT AYVKNFA... .....DNKWY YFDDSRVTET A.......... ..........  ..........  .PENSIAGSA YLLFYIRRHK
   P40818 LKK YNLFSVSNHY G.GLDGGHYT AYCKNAA... .....RQRWF KFDDHEVSDI S.......... ..........  ..........  .VSSVKSSAA YILFYTSLGP
   P25037 PLT YSLRSVIVHY G.THNYGHYI AFRKYRG... .......CWW RISDETVYVV DEAEV..... ..........  ..........  ....LSTPGV FMLFYEYDFD
   Q01476 EYG YSLFSVFIHR G.EASYGHYW IYIKDRNRN. ....  GIWR KYNDETISEV QEEEVFNFNE ..........  ..........  ....GNTATP YFLVYVKQGQ
 Z47811x3 QHK YELHAIIVHS G.EANRGHYW TYKLKKSID. ....GLEEWE KLNDQNADRV DWPKVESDSF GTGS......  ..........  ... RDAPSA YMLMYVRSDA
CONSENSUS      Y L gV  H  G     GHYt  O k              WO  ODD                                                A YOLfY
                 a                 O
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Fig. 2. Compilation of the cysteine (**A**) and histidine (**B**) conserved domains of HAUSP and 20 members of the ubiquitin protease protein family. Proteins were detected by homology to HAUSP by the Blast program and the domain sequences were aligned using Pileup (GCG) before manual editing. The origins of the proteins are shown in (A), and the sequences are presented in the same order in (B). Further details of the selected proteins may be obtained from the quoted accession numbers. The consensus shows highly conserved residues that are present in HAUSP in bold; those that are not present in HAUSP are shown in lower case. Positions where hydrophobic residues are conserved are indicated by O. Note that there are groups of proteins which are more highly related to each other than to the family as a whole.

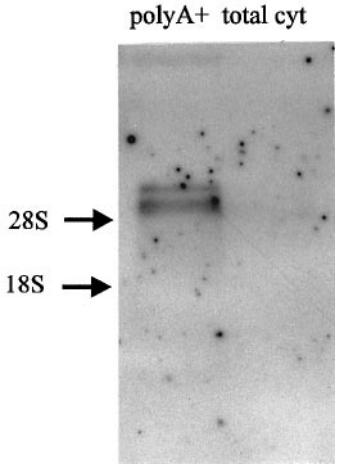


Fig. 3. Northern blot of the HAUSP transcript. Poly(A)$^+$ selected and total cytoplasmic RNA samples were probed with RT–PCR clone MRMF15. The positions of the 28S and 18S ribosomal RNAs were determined in comparison with the stained gel and probing with a clone containing 28S rRNA sequences.

deleting sequences that have been implicated in HAUSP binding (Meredith *et al.*, 1994, 1995). Probing of this blot with r29 serum clearly indicated that the radiolabelled, co-immunoprecipitated band was indeed HAUSP (Figure 5A). A control probing the same blot with an anti-Vmw110 rabbit serum showed that Vmw110 expression and immunoprecipitation was equivalent in all three virus infections (Figure 5C).

In a converse experiment, r29 serum immunoprecipitated HAUSP from both uninfected and infected cells (Figure 5E). Probing the Western blot of these immunoprecipitation products with Mab 11060 showed that wild-type, but not mutant D12 or E52X Vmw110, was co-precipitated with HAUSP (Figure 5D). A control blot of the supernatants after the immunoprecipitations confirmed that infection and Vmw110 expression were equivalent in the three infected cell samples (Figure 5F). The amount of HAUSP precipitated from wild-type virus-infected cell extracts was reproducibly decreased compared with the other samples, indicating that the HAUSP epitopes recognized by r29 serum may be partially masked by the binding of Vmw110. These results confirm the specificity of r29 serum in immunoprecipitation reactions, and incontrovertibly establish that Vmw110 binds to the protein

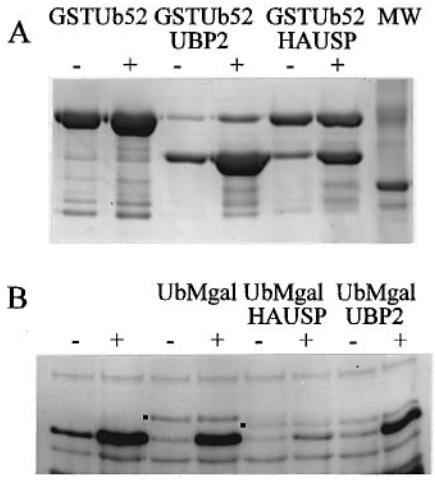


Fig. 4. Cleavage of model ubiquitin fusion protein substrates by HAUSP. (**A**) Bacteria harbouring plasmids expressing the indicated proteins (see Materials and methods) were grown up and expression of HAUSP or the positive control yeast UBP2 enzyme was either induced or not by the addition of IPTG (lanes marked + and – respectively). MW indicates molecular weight markers, the upper band (ovalbumin; 45 kDa) co-migrating with the predicted 42 kDa GST–Ub52 fusion protein, while the most prominent band in the MW lane is carbonic anhydrase, 29 kDa. The predicted size of the observed GST–Ub specific cleavage product is 36 kDa. (**B**) A Western blot of β-galactosidase proteins expressed by bacteria harbouring plasmids expressing the Ub–Met–β-gal model substrate and either HAUSP or UBP2, as marked. Expression of the USP enzymes in parallel cultures was either induced by the addition of IPTG or left uninduced (as indicated by + and –). The position of the uncleaved substrate is indicated by a dot in the second pair of tracks, while the correctly cleaved Met–β-gal product (as defined by the UBP2-positive control in the fourth pair of tracks) is indicated by a dot next to the third pair. Constitutive expression of UBP2 in this experiment is sufficient to cleave completely the low levels of substrate present. The more prominent and highly induced band of slightly higher gel mobility than the Met–β-gal product band is the truncated form of β-galactosidase expressed by the Novagen Blue (DE3) bacteria.

expressed by the cloned cDNA. It is worth noting that in some r29 immunoprecipitation experiments, trace amounts of mutant D12 Vmw110 were detected (data not shown, but it is extremely faintly visible in Figure 5D); perhaps the high affinity of Mab 11060 for Vmw110 gives a more sensitive assay, or the r29 antibodies stabilize a weak HAUSP–D12 interaction. This result suggests that the Vmw110 sequences which contact HAUSP have not been completely removed by the D12 deletion, which might explain why a virus with the D12 deletion grows more efficiently than a virus with the E52X deletion (Meredith *et al.*, 1995). Further virus deletion mutants are currently under construction to investigate in more detail the precise Vmw110 sequence requirements for HAUSP binding.

The r29 immunoprecipitation experiments reproducibly precipitated not only HAUSP but also a band of slightly higher molecular weight. It is possible that the larger band is an isoform of HAUSP, perhaps translated from the relatively minor higher molecular weight mRNA (Figure 3), or it could be a form with altered post-translational modifications.

HAUSP is associated with a subset of ND10

Since Vmw110 binds to HAUSP and also localizes within the nucleus at the PML-containing ND10 structures, we investigated by immunofluorescence whether HAUSP was a normal component of ND10. Initial experiments used r29 serum, but consistent results of higher quality were obtained with r206 serum which was generated after immunization with a branched peptide containing HAUSP residues 1087–1102. Hep2 and human fetal lung (HFL) cells were co-stained with r206 and Mab 5E10 which detects PML (Stuurman *et al.*, 1992; Dyck *et al.*, 1994). The anti-HAUSP r206 serum gave a microspeckled nuclear staining pattern, excluding the nucleoli, with a small and variable number of brighter dots in some (but not all) cells, some of which co-localized with PML in ND10 (this was more easily seen in Hep2 cells) (Figure 6A–D). As a control, the corresponding pre-immune r206 serum gave very faint staining that was not concentrated in the nucleus (not shown), and which indicates that the staining pattern was specific for HAUSP and that channel overlap during fluorescence was undetectable. Additional confirmation of the specificity of r206 serum was obtained in combined immunoprecipitation and Western blotting experiments (analogous to Figure 5A–C) and by immunofluorescence of cells transfected with a HAUSP expression vector (data not shown). From these results we can conclude that HAUSP is a predominantly nuclear protein which is present in a minority of ND10. This suggests that there is a dynamic interaction between HAUSP and ND10 which may depend on some aspect of the status of the cell.

There is precedence for a dynamic interaction of a protein with ND10 in that NDP52, while present in ND10 in some cell lines, only becomes associated with ND10 in HeLa and Cos cells after treatment with interferon (Korioth *et al.*, 1995). It remains to be seen if the localization of HAUSP at ND10 can also be modulated by cytokines or other stimuli.

Co-localization of HAUSP, Vmw110 and ND10 at early times of virus infection

To investigate the effect of Vmw110 on the intranuclear distribution of HAUSP, HFL cells were infected with wild-type HSV-1 strain 17 and mutant derivative FXE. Virus FXE expresses a mutant form of Vmw110 lacking the RING finger domain (Everett, 1989), which eliminates its ability to disrupt ND10 but not its migration to ND10, thus resulting in a stable co-localization of Vmw110 and PML (Everett and Maul, 1994; Maul and Everett, 1994). In contrast, in a wild-type virus infection, Vmw110 transiently co-localizes with PML before the disruption of ND10 (Maul and Everett, 1994). Infected cells were co-stained with r206 serum to detect HAUSP and with either Mab 5E10 to detect PML or Mab 11060 to detect Vmw110. In a proportion of wild-type virus-infected cells, some r206 staining co-localized with PML (Figure 6E and F; most easily seen in the lower cell) and examination of large numbers of infected cells suggested that this partial co-localization was more extensive than that seen in uninfected cells (compare with Figure 6C and D). However, there was considerable variability from cell to cell, and the images have been selected to give a fair representation of this variability. When wild-type virus-infected cells were co-stained with r206 and Mab 11060, careful examination of the distribution of increased localized r206 staining in a proportion of the cells again

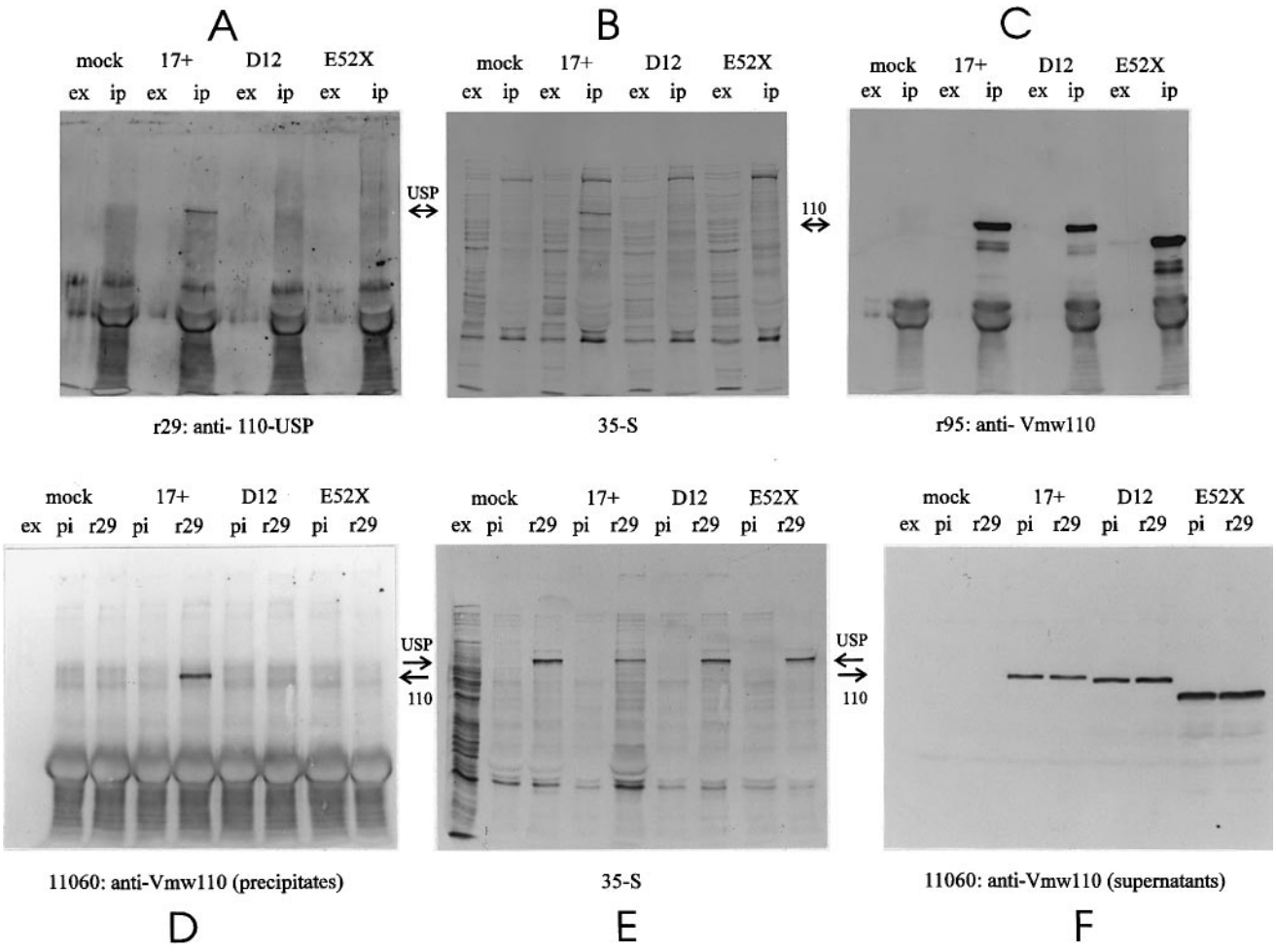
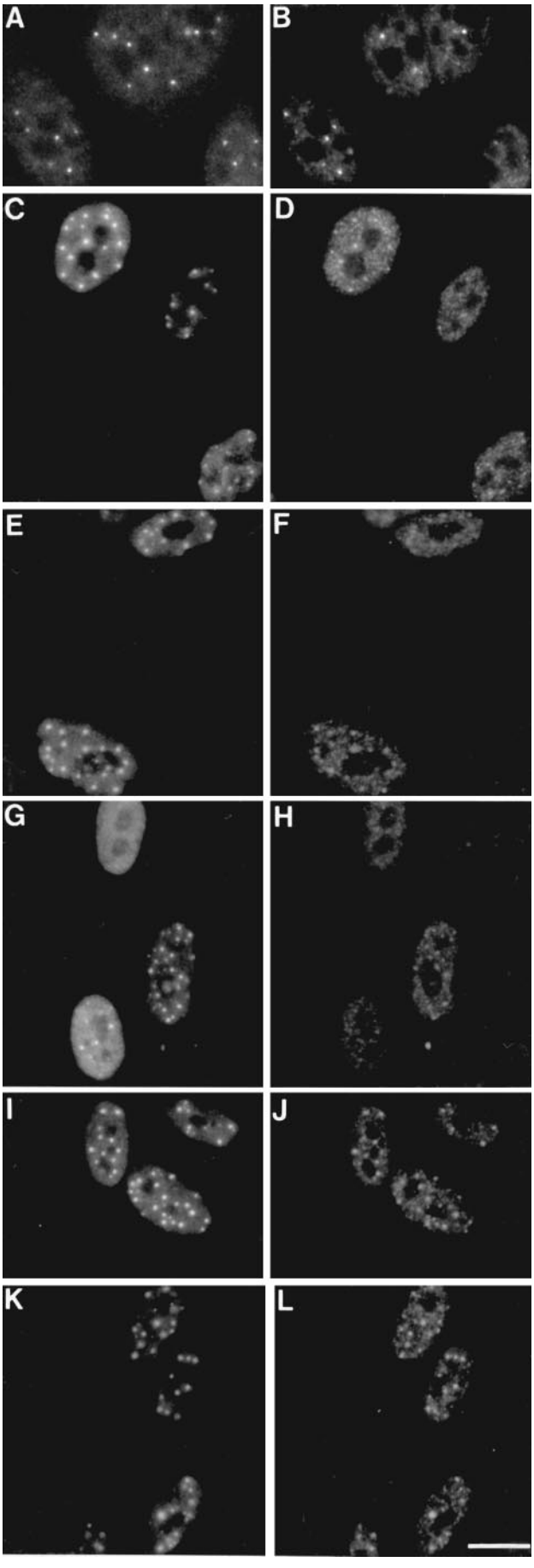


Fig. 5. Vmw110 co-immunoprecipitates from virus-infected cells in complex with HAUSP. Immune precipitates using anti-Vmw110 Mab 11060 were prepared from mock-infected HeLa cells or cells infected with viruses 17+, D12 or E52X as indicated. The samples were Western blotted and probed with r29 serum (**A**). The blots were stripped and re-probed with anti-Vmw110 rabbit serum r95 to detect precipitated Vmw110 (**C**), then stripped again to detect labelled proteins by autoradiography (**B**). The HAUSP and Vmw110 proteins are arrowed. ex indicates tracks containing samples of the radiolabelled extracts used for the immunoprecipitations [which contain too little protein for HAUSP to be detected by r29 serum in (A)]; ip indicates tracks containing the immunoprecipitates. In a converse experiment, r29 serum was used to precipitate HAUSP from extracts of uninfected and infected cells prepared exactly as described above (lanes marked r29). The corresponding pre-immune serum was used as a control (lanes marked pi). Lanes marked ex contain labelled proteins from uninfected cells as a marker. After Western blotting, precipitated Vmw110 was detected with Mab 11060 (**D**); the blot was then stripped to detect radiolabelled proteins (**E**). The supernatants from the precipitations were analysed by Western blotting using Mab 11060 as a control for infection and expression of Vmw110 (**F**).

indicated the presence of HAUSP in the Mab11060-stained nuclear dots (rightmost cell, Figure 6G and H). Although more extensive than the co-localization of HAUSP and PML in uninfected cells, this effect was by no means general and was most easily observed when Vmw110 was present in small amounts in discrete dots, which presumably identifies cells at the earliest stages of infection. As infection proceeds, Vmw110 accumulates and becomes increasingly diffuse within the nucleus, which makes visualization of any co-localization of Vmw110 and HAUSP more difficult or even impossible (upper and lower cells, Figure 6G and H). In contrast, cells infected with virus FXE showed striking co-localization of PML and HAUSP (Figure 6I and J) and Vmw110 and HAUSP (Figure 6K and L) in a very high proportion of the nuclear dots in all infected cells. Control experiments using the monoclonal antibodies and the pre-immune r206 serum indicated that these observations were specific and not subject to channel overlap effects (not shown).

Confirmation of the above conclusions was obtained using confocal microscopy of uninfected and virus FXE-infected Hep2 cells (Figure 7). In uninfected cells, merging of the PML and HAUSP signals clearly indicated that HAUSP co-localized with PML in a proportion of ND10. The greater resolution of these images clearly confirmed the presence of localized accumulations of HAUSP which were not in ND10 (the red dots in the merged image, top row Figure 7). In virus FXE-infected cells, the great majority of ND10 (as defined by PML accumulations) also contained HAUSP (Figure 7, middle row), and there was also extensive co-localization of FXE-mutant Vmw110 and HAUSP (Figure 7, bottom row). Differences in the extent of infection of the cells in this experiment result in varying levels of Vmw110 expression and therefore variation of the colour balance of the cells in the merged image.

These results indicate that HAUSP has a transient association with ND10 in uninfected cells, but that its association with Vmw110 at early times of infection leads to an increased proportion of ND10 which contain



detectable HAUSP. Because the biochemical data indicate such a strong interaction between HAUSP and Vmw110, this conclusion is not surprising. However, it is difficult to quantify these effects because of the dynamic nature of the situation during wild-type virus infection, in which Vmw110 transiently co-localizes with PML, and then disrupts ND10. However, in FXE infections the interactions appear to be frozen, so that the great majority of ND10 contain PML, HAUSP and Vmw110.

A relevant consideration is the localization of HAUSP during infection with a virus which expresses a binding-defective form of Vmw110 such as D12. This is not a straightforward question because of the variability seen between cells in wild-type virus infection (as discussed above) and the localization of HAUSP within a subset of ND10 in the absence of Vmw110. However, careful examination by immunofluorescence of cells infected with virus D12 revealed no greater co-localization of HAUSP with PML than in uninfected cells (data not shown). Due to the nature of the assay, this conclusion must be treated with caution but, if true, it has an interesting consequence because the D12 deletion also reduces the ability of Vmw110 to disrupt ND10 during virus infection (Meredith *et al.*, 1995). This implies that the presence of both HAUSP and an active form of Vmw110 is required for the disruption of ND10, which implicates a ubiquitin-dependent pathway in this process. This is particularly intriguing since a ubiquitin-homology family protein has been identified which binds to PML and is located in ND10 (Boddy *et al.*, 1996), and restoration of normal differentiation of promyelocytic leukaemic blasts following retinoic acid treatment correlates with the destruction of the PML–RARα fusion protein by a proteasome-dependent (and therefore probably a ubiquitin-dependent) pathway (Yoshida *et al.*, 1996).

Discussion

Ubiquitin-dependent pathways have been shown to play crucial roles in several cellular processes, among which are regulation of gene expression, control of the cell cycle, DNA repair and differentiation (reviewed by Hochstrasser, 1995; Wilkinson, 1995). One of the best understood examples of a ubiquitin-dependent pathway involved in the control of gene expression is the activation of NFκB.

Fig. 6. HAUSP co-localizes with PML in a subset of ND10 in uninfected cells and more generally after infection. Uninfected Hep2 cells (**A** and **B**) and HFL cells (**C** and **D**) were co-stained with anti-PML Mab 5E10 (left-hand panels) and anti-HAUSP serum r206 (right-hand panels). In panels (E) to (L), HFL cells were infected with wild-type HSV-1 strain 17 (**E**–**H**) or Vmw110 RING finger mutant FXE (**I**–**L**), fixed 1 h after virus absorption and stained with anti-PML Mab 5E10 (**E** and **I**) or anti-Vmw110 Mab 11060 (**G** and **K**), simultaneously with anti-HAUSP serum r206 (right-hand panels). The left- and right-hand panels show the same fields of cells, which have been selected to illustrate the range of phenotypes observed after examination of several thousand cells. Although it was possible to find cells in the experiments illustrated in panels (E–H) which exhibited far more striking co-localization than the examples shown, these particular fields were selected as being a fair representation of the bulk of the population. Careful examination is required to confirm that most (but not all) the regions of greater anti-HAUSP staining co-localize with some (but not all) of the punctate anti-PML or anti-Vmw110 staining. The bar in panel (L) indicates 5 μm.

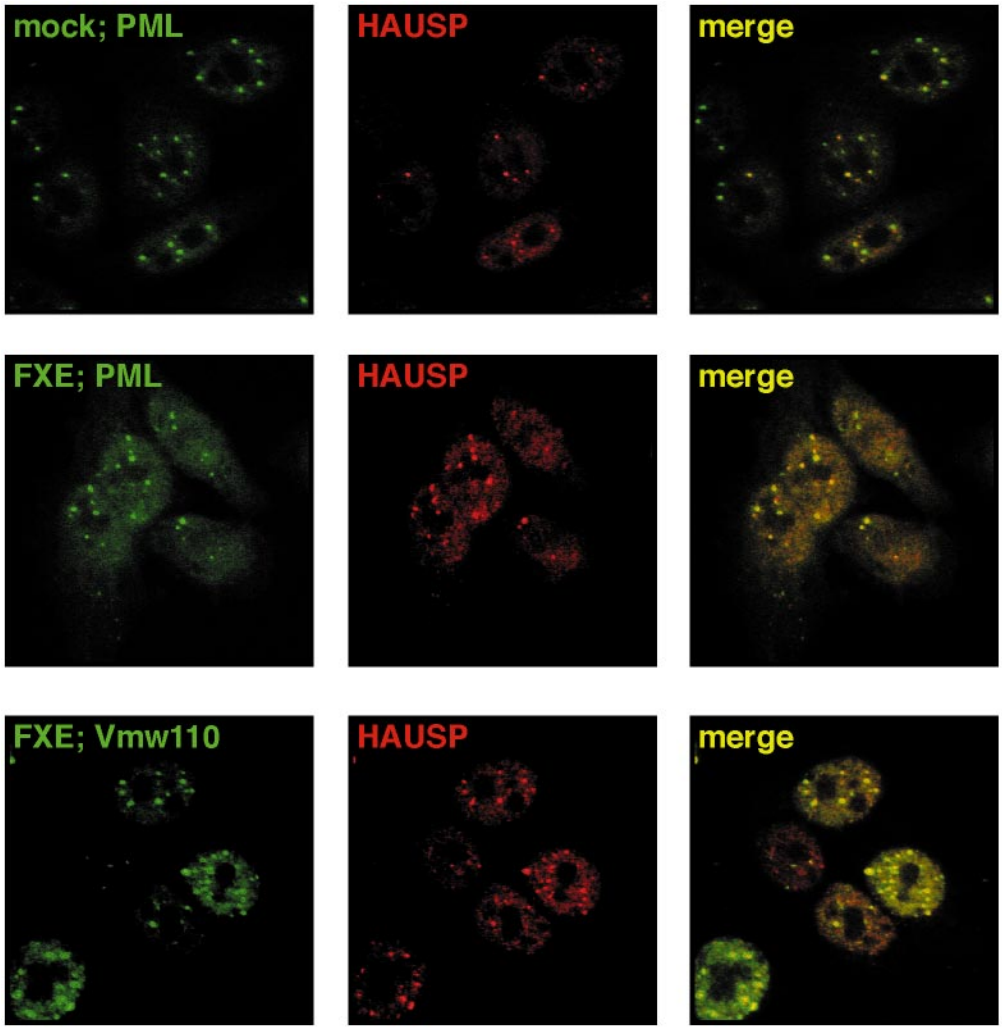


Fig. 7. Confocal microscopy analysis of the presence of HAUSP in ND10 in uninfected mutant virus FXE-infected Hep2 cells. The samples were prepared as described in Materials and methods and the detection of PML, Vmw110 and HAUSP is indicated by the relevant coloured labels.

This involves two distinct processes, first, the cleavage of the p105 precursor of the p50 subunit of NFκB requires ubiquitin conjugation (Palombella *et al.*, 1994), and secondly the destruction of IκB (a process which allows NFκB to migrate to the nucleus in an active form) requires ubiquitination of the inhibitor in a phosphorylation-dependent manner (Scherer *et al.*, 1995). During the cell cycle, cyclin B is targeted for ubiquitination and destruction to allow progression from metaphase to anaphase, and the components for this process are associated with the spindle itself (reviewed by Murray, 1995). Repair of UV-damaged DNA in yeast requires the rad6 protein, a ubiquitin-conjugating enzyme which is targeted to single-stranded DNA by complex formation with the rad18 protein (Bailly *et al.*, 1994). These latter two examples illustrate that the activity or specificity of a ubiquitination process may be modulated not only by biochemical mechanisms, but also by interactions with proteins which modify the intracellular localization of the ubiquitinating activity. There is an obvious parallel with the observation that Vmw110 can increase the proportion of ND10 which contain HAUSP (Figures 6 and 7).

The examples cited above concern the control of protein stability by directed ubiquitination of specific substrates, while HAUSP is a predicted ubiquitin-specific protease. USP enzymes are characterized by the presence of two conserved active site domains (Figure 2) and several have been shown to cleave ubiquitin from model substrates such as Ub-Met-β-galactosidase by hydrolysing the bond between the C-terminal double glycine of ubiquitin and the linking methionine residue. We have found that HAUSP is indeed active in this assay, and is therefore a true USP (Figure 4). USP enzymes fall into at least two classes. The first includes proteins involved in the generation of free ubiquitin from precursor fusion proteins or from peptide-linked polyubiquitin after proteolysis of the substrate by the proteasome (e.g. yeast doa4; Papa and Hochstrasser, 1993). The second comprises an increasing number of de-ubiquitinating proteins which may recognize and stabilize specific substrates by removing ubiquitin adducts. Examples of this class include the *Drosophila fat facets* protein, whose de-ubiquitinating activity is required for proper development of the eye (Huang *et al.*, 1995), and DUB-1, a cytokine-inducible immediate-early

gene which regulates cell growth (Zhu *et al.*, 1996). The important concept here is that ubiquitin-specific protease enzymes can be substrate-specific and involved in highly regulated processes. The large size of HAUSP suggests that it may recognize and bind to specific proteins, and either its substrate specificity or activity could be modified by the binding of Vmw110.

Modulation of ubiquitin-dependent pathways has been shown to play a role in a number of virus infections. For example, HPV E6 protein directs the ubiquitination and destruction of p53 to prevent apoptosis (Scheffner *et al.*, 1993) while African swine fever virus encodes a component of the ubiquitin-conjugating pathway (Hingcamp *et al.*, 1992; Rodriguez *et al.*, 1992). Our observations suggest that HSV-1 interacts with an uncharacterized ubiquitination pathway to stimulate viral gene expression and infectivity. A simple scenario is that viral proteins synthesized at the onset of infection may be recognized as foreign by the cell and targeted for degradation by ubiquitination. Vmw110 may be stimulating or re-directing the activity of HAUSP to de-ubiquitinate and therefore stabilize viral proteins. This would be consistent with the inefficient onset of viral replication in the absence of Vmw110, the observation that this defect can be overcome in high multiplicity infections, and the synergistic activation of gene expression induced by Vmw110 and the major viral transactivator, Vmw175, in co-transfection experiments (Everett *et al.*, 1991). It is intriguing that viral 'replication compartments' (sites of accumulation of replicated viral DNA and replication proteins) are preferentially located in close proximity to ND10 (Maul *et al.*, 1996), and therefore close to the increased concentrations of HAUSP imported into ND10 in association with Vmw110. An alternative (but not exclusive) hypothesis is that Vmw110 is modifying the activity of HAUSP on cellular targets, thereby either increasing or decreasing their stability by inhibiting or activating deubiquitination. As discussed above, it is likely that the dispersal of ND10 during the early stages of virus infection requires both HAUSP and an active form of Vmw110 and therefore it is possible that substrates for HAUSP are found at ND10.

The finding that Vmw110 binds strongly and specifically to a ubiquitin-specific protease implicates a novel mechanism in the efficient initiation of viral gene expression. Elucidation of the cellular pathways in which HAUSP is involved may lead to greater understanding of the balance between HSV latency and lytic replication, and how this is regulated in conjunction with cellular control mechanisms.

Materials and methods

Purification of the Vmw110-associated 135 kDa protein

Glutathione–agarose beads were incubated with bacterial extracts containing a GST fusion protein containing residues 594–775 of Vmw110 as described (Meredith *et al.*, 1995). A 4 ml aliquot of a 50% slurry of the beads was incubated sequentially with batches of whole-cell HeLa extracts made from a total of ~5×10^9 cells. The extracts had been precleared by incubation with a similar volume of beads charged with GST alone to reduce background binding. The beads were thoroughly washed with 50 mM Tris–HCl pH 8.0, 0.5 M NaCl, 1 mM EDTA, 0.5% NP-40 and the complexes of fusion protein with bound HeLa cell proteins were eluted in 4 ml with reduced glutathione (50 mM in 0.25 M Tris–HCl pH 7.0). The eluate was concentrated to 0.2 ml by Centricon centrifugation and the proteins were separated on a 7.5% SDS–polyacrylamide gel. Pilot experiments determined that the 135 kDa protein could be visualized by staining, and that it was separated from any contaminating bacterial proteins. As an aid to identification, a sample of 135 kDa protein prepared on a smaller scale from a radiolabelled extract was added to the preparation before electrophoresis. Proteins were transferred from the gel to a Problott membrane and visualized by Ponceau S staining and autoradiography. The appropriate band was excised and submitted for peptide sequence analysis after cleavage *in situ* with *Achromobacter* protease, which cuts after lysine residues.

Cloning of a family of cDNAs encoding the 135 kDa protein

Of the six peptide sequences that were obtained, several had too many unknown, arginine, leucine or serine residues to allow the design of primers or probes of reasonable redundancy. Eventually, a 53mer oligonucleotide based on peptide 45 was designed using codon usage and CpG depletion assumptions for hybridization screening of a HeLa cell cDNA library made by insertion of randomly primed cDNAs into lambda ZAPII. The library was plated at a density of 5×10^4 plaques per 140 mm dish and duplicate filter lifts were screened by hybridization to ^{32}P-end-labelled oligonucleotide using hybridization conditions which retained faint non-specific signals. Candidate plaques giving above-background signals on both filters were picked, re-screened in the same manner, and small cultures of the positive clones were prepared. The bacteriophage DNAs were prepared and screened by Southern blotting using the same probe at increasing hybridization temperatures; the cDNA inserts of those retaining the most stringent signals were excised and analysed by DNA sequencing. Two partially overlapping clones were identified which contained a sequence corresponding to the probe, and one of them also contained the coding sequences for peptides 52/2 and 52. A PCR fragment derived from this clone was used to re-screen the original library and a total of 10 further positive clones were isolated from another 1×10^5 plaques. Multiple overlapping restriction enzyme fragments were prepared from a selection of these clones and their DNA sequence revealed the 5′ untranslated region and the N-terminal 1046 codons. Peptide sequence 39 detected a match with an entry in the dbest database (Lennon *et al.*, 1996); the relevant plasmid (pT80922) was sequenced to reveal the 3′ end of the open reading frame. Primers were designed from the lambda cDNA and pT80922 plasmid clones and used in RT–PCR with HeLa cell RNA, and a clone was obtained which linked the overlapping cDNA clones with the pT80922 3′ end clone. The authenticity of this junction was confirmed by RT–PCR using a different primer combination. The final DNA sequence extends for 4022 bp; taking into account the estimated size of the major transcript detected on Northern blots (Figure 3) and the poly(A) tail it appears that ~200–300 bp of untranslated 5′ leader remain to be isolated. The available DNA and translated peptide sequence of HAUSP is available on Accession Number Z72499.

USP cleavage assays

Cleavage assays were conducted using T7-driven IPTG-inducible HAUSP expression plasmids. These were constructed by linkage of appropriate fragments from the overlapping cDNAs to the 3′ end clone pT80922 (see above) by a short segment derived by RT–PCR. After introduction of an *Nde*I site at the presumed ATG by PCR mutagenesis, the complete HAUSP coding sequence was inserted into the T7 expression plasmid pET3a (a pBR322 Amp^r replicon) using the *Nde*I site to place the initiating ATG at the optimal position (plasmid pT7–HAUSP). A plasmid containing the T7–HAUSP expression cassette in a pACYC184 Cm^r replicon was constructed by inserting a *Sal*I–*Cla*I fragment from pT7–HAUSP between the *Sal*I and *Cla*I sites of pACYC184 (pACT7–HAUSP). A plasmid expressing the GST–Ub52 fusion protein substrate (PGEX–Ub52) was constructed by in-frame linkage of a human Ub52 (Baker and Board, 1991) cDNA (kindly provided by A.Ishov and G.Maul, The Wistar Institute, Philadelphia) to a plasmid based on pGEX2T (pBR322 Amp^r replicon). Plasmid pAC–M–β-gal expresses the Ub–Met–β-gal fusion protein substrate in a pACYC184 Cm^r replicon, and plasmid pRB105 expresses the yeast UBP2 USP enzyme from an IPTG-inducible tac promoter in a pKK-based plasmid (pBR332 Amp^r replicon); both were kindly provided by R.T.Baker (Baker *et al.*, 1992). The complete promoter, UBP2 coding and transcription termination signal region was excised from pRB105 on a *Sph*I–*Sca*I fragment and inserted between the *Sph*I and *Eco*RV sites of pACYC184 to create pACYC–UBP2 (pACYC184 Cm^r replicon). For cleavage of the GST–Ub52 substrate, *E.coli* strain BL21(DE3) bacteria harbouring pGEX–Ub52 were transfected with either pACT7–HAUSP or pACYC–UBP2, and Amp^r Cm^r colonies were grown up, induced with IPTG and soluble

protein extracts prepared by sonication. GST fusion proteins were purified by binding to glutathione–agarose beads and analysed by Coomassie staining of SDS–polyacrylamide gels. For cleavage of the Ub–Met–β-gal substrate, *E.coli* strain Novagen Blue (DE3) bacteria harbouring pAC–M–β-gal were transfected with either pT7–HAUSP or pRB105, and Amp^r Cm^r colonies were grown up, induced with IPTG and total protein extracts were analysed by Western blotting using anti-β-galactosidase rabbit polyclonal antibody r12741 (kindly provided by H.Marsden, MRC Virology Unit).

Northern blotting

Poly(A)$^+$ RNA was prepared from HeLa cells using the Invitrogen Fast Track mRNA isolation kit and total cytoplasmic HeLa cell RNA was prepared by phenol and chloroform extraction. Samples (10 μg of poly(A)$^+$ and 10 μg of total cytoplasmic RNA) were electrophoresed through 1.2% agarose–formaldehyde gels and the separated RNAs were transferred to Genescreen Plus membrane. Hybridization was performed in a buffer containing 5× SSPE, 50% formamide, 5× Denhart's solution, 1% SDS at 42°C overnight, using probe 1411 (including 30 bp of 5′ untranslated leader and HAUSP codons 1–491) or probe MRMF15 (HAUSP codons 424–1084). The filter was stripped and subsequently hybridized to a γ-actin probe. The positions of the 28S and 18S ribosomal RNAs were established in comparison with the stained gel and by hybridization with a probe containing 28S sequences.

Viruses and cells

HSV-1 strain 17 syn$^+$ was the wild-type strain used in these studies. The FXE, D12 and E52X viruses with defined deletions in Vmw110 have been described previously (Everett, 1989; Meredith *et al.*, 1995). All viruses were grown and titrated in baby hamster kidney clone 13 (BHK) cells propagated in Glasgow Modified Eagle's Medium (GMEM) containing 100 units/ml penicillin and 100 μg/ml streptomycin, and supplemented with 10% newborn calf serum and 10% tryptose phosphate broth. HeLa cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 2.5% fetal calf and 2.5% newborn calf sera and antibiotics as above. Human fetal lung (HFL) cells (Flow Laboratories) and Hep2 cells were grown in GMEM supplemented with 10% fetal calf serum and antibiotics as above.

Antibodies and immunoprecipitations

A GST fusion protein including HAUSP residues 28–427 was expressed in *E.coli*, purified by gel electrophoresis and used to immunize rabbits. A serum was obtained (r29) which detected a protein of ~135 kDa (determined in comparison with known proteins) in Western blots of uninfected HeLa cell extracts. The identity of this protein as HAUSP was confirmed as described in the text. Serum r206 was generated by immunization of rabbits with branched chain peptides including residues 1087–1102 of HAUSP (see Figure 1).

HeLa cells in 80 mm dishes were pre-labelled with 500 μCi [^{35}S]methionine; the labelling medium was then removed and the cells were infected in normal medium with HSV-1 strain 17 and mutant viruses D12 (deletion of Vmw110 residues 594–633) or E52X (deletion of Vmw110 residues 594–775). A multiplicity of 10 p.f.u. per cell was used in all cases. Extracts were prepared 16 h post-infection and immunoprecipitations were conducted with anti-Vmw110 Mab 11060 (Everett *et al.*, 1993) exactly as described (Meredith *et al.*, 1994). Precipitated proteins were separated on 7.5% glycine SDS gels, the proteins were transferred to nitrocellulose filters by Western blotting, and probed with r29 serum using the Amersham ECL system. The blots were stripped and re-probed with anti-Vmw110 rabbit serum r95 (Everett *et al.*, 1993) to detect precipitated Vmw110, then stripped again to detect labelled proteins by autoradiography. Immunoprecipitations with r29 serum were conducted in the same manner, except that precipitations with the pre-immune serum were run in parallel as controls. After Western blotting, precipitated Vmw110 was detected with Mab 11060, then the blot was stripped to detect radiolabelled proteins. The supernatants from the precipitations were analysed by Western blotting using Mab 11060 as a control for infection and expression of Vmw110.

Immunofluorescence

Human fetal lung (HFL) cells were infected with wild-type HSV-1 strain or viruses expressing the D12 (deletion of residues 594–633) or FXE (deletion of residues 106–150) mutant forms of Vmw110 at a multiplicity of 5 p.f.u. per cell. The infections were allowed to proceed for 1 h after a 1-h absorption period; the cells were then fixed with formaldehyde (5%, v/v, in PBS containing 2% sucrose) and permeabilized with 0.5% NP-40 in PBS with 10% sucrose. The primary antibodies were diluted in PBS containing 1% newborn calf serum. Anti-Vmw110 Mab 11060 was used at a dilution of 1/2000, anti-PML Mab 5E10 was used at 1/20 and anti-HAUSP r206 serum was used at 1/200. Goat anti-mouse FITC-labelled and goat anti-rabbit TRITC-labelled secondary antibodies (Sigma) were used at 1/100. After staining, the coverslips were mounted and examined in a Nikon Microphot-SA microscope with an oil immersion ×60 objective lens and appropriate filters.

Confocal microscopy

Coverslips of Hep2 cells were prepared as described above, then examined using a Nikon microscope with a ×60 1.4 oil immersion lens combined with a Noran Odessey confocal laser. For FITC fluorescence, the excitation was at 488 nm and emission at 515 nm; for TRITC, fluorescence excitation was at 529 nm and emission at 550 nm. The images were captured using Metamorph software and the FITC and TRITC image pairs were merged using Photoshop.

Acknowledgements

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