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Specific destruction of kinetochore protein CENP-C and disruption of cell division by herpes simplex virus immediate-early protein Vmw110

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Examination of cells at the early stages of herpes simplex virus type 1 infection revealed that the viral immediate-early protein Vmw110 (also known as ICP0) formed discrete punctate accumulations associated with centromeres in both mitotic and interphase cells. The RING finger domain of Vmw110 (but not the C-terminal region) was essential for its localization at centromeres, thus distinguishing the Vmw110 sequences required for centromere association from those required for its localization at other discrete nuclear structures known as ND10, promyelocytic leukaemia (PML) bodies or PODs. We have shown recently that Vmw110 can induce the proteasome-dependent loss of several cellular proteins, including a number of probable SUMO-1-conjugated isoforms of PML, and this results in the disruption of ND10. In this study, we found some striking similarities between the interactions of Vmw110 with ND10 and centromeres. Specifically, centromeric protein CENP-C was lost from centromeres during virus infection in a Vmw110- and proteasome-dependent manner, causing substantial ultrastructural changes in the kinetochore. In consequence, dividing cells either became stalled in mitosis or underwent an unusual cytokinesis resulting in daughter cells with many micronuclei. These results emphasize the importance of CENP-C for mitotic progression and suggest that Vmw110 may be interfering with biochemical mechanisms which are relevant to both centromeres and ND10.

Keywords: CENP-C/centromere/HSV-1/kinetochore/Vmw110

Introduction

Recent studies have revealed that herpes simplex virus type 1 (HSV-1), a ubiquitous pathogen, interacts in specific ways with subnuclear structures during infection. The 152 kb viral genome encodes at least 76 genes, of which five encode immediate-early (IE) regulatory proteins. The product of gene IE-1, Vmw110 (also known as ICP0), has a central role in virus–cell interactions. During primary infection of an individual, HSV-1 undergoes lytic replication in epithelia, then establishes a latent infection in neurones. Previous studies have shown that Vmw110 plays an important role in the activation of viral gene expression during lytic infection, and it has been implicated in efficient reactivation from latency (for reviews, see Everett *et al*., 1991; Fields *et al*., 1996). Exactly how the protein accomplishes these functions is not known, but recent studies showing that Vmw110 induces the proteasome-dependent degradation of several cellular proteins suggest that changes in the intranuclear environment may be involved (Everett *et al*., 1998a).

Vmw110 has an intriguing association with distinct nuclear structures known as ND10, promyelocytic leukaemia (PML) bodies or PODs. There are usually between five and 30 ND10 domains per cell, each of which contains a number of proteins (reviewed by Sternsdorf *et al*., 1997a; Maul, 1998). The best studied ND10 protein is PML, called this because most cases of acute promyelocytic leukaemia (APL) are associated with a chromosomal translocation which results in expression of a fusion protein comprising parts of normal PML and the retinoic acid receptor (RAR) α (de The *et al*., 1991; Goddard *et al*., 1991; Kakizuka *et al*., 1991; Kastner *et al*., 1992; Pandolfi *et al*., 1992). Expression of the PML–RARα fusion protein causes disruption of ND10, whereas treatment of APL cells with retinoic acid results in degradation of the fusion protein, re-assembly of normal ND10 and restored differentiation of the transformed cells (Dyke *et al*., 1994; Koken *et al*., 1994; Weiss *et al*., 1994; Yoshida *et al*., 1996).

The normal functions of ND10 structures are not well understood, but they respond to stress, interferon treatment and infection with several DNA viruses (for references, see Everett *et al*., 1997). Parental HSV-1 genomes preferentially migrate to the periphery of ND10 (Maul *et al*., 1996), then newly expressed Vmw110 concentrates in these domains which are disrupted within a few hours as a consequence of Vmw110-induced proteasome-dependent degradation of several PML isoforms (Maul *et al*., 1993; Everett and Maul, 1994; Maul and Everett, 1994; Everett *et al*., 1998a). The affected PML isoforms are most likely those conjugated to the ubiquitin-like protein SUMO-1 (Sternsdorf *et al*., 1997b; Kamitani *et al*., 1998; Muller *et al*., 1998). Vmw110 can also affect the stability of a number of other uncharacterized SUMO-1-conjugated proteins (Everett *et al*., 1998a), and a further indication that the mechanism of action of Vmw110 might involve changes in protein stability came from detection of its strong interaction with HAUSP, a ubiquitin-specific protease (Everett *et al*., 1997).

Because incoming viral genomes preferentially are shutdown or repressed in the absence of Vmw110 (Preston and Nicholl, 1997; Samaniego *et al*., 1998), it is tempting to speculate that the action of Vmw110 in promoting virus

infection and reactivation from quiescence may be caused by the targeted destruction of one or more nuclear proteins that are involved in the repression of viral gene expression (Everett *et al*., 1998b). One classical example of a cellular repression mechanism is that associated with heterochromatin, a specialized type of chromatin in which gene expression can be stably repressed (reviewed by Elgin, 1996). We were therefore particularly interested to note that Vmw110 could also accumulate at punctate foci which were not ND10 and which underwent striking and characteristic changes in distribution during the early stages of infection of cells undergoing mitosis. The aim of the present study was to determine the nature of these structures, the sequences of Vmw110 that were responsible for this distribution and the biochemical and cellular consequences of the interaction. We found that Vmw110 binds to centromeres in both mitotic and interphase cells and induces the rapid loss of the centromeric protein CENP-C (but not CENP-B). The proteasome-dependent degradation of CENP-C culminated in a profound disruption of kinetochore structure and resulted in striking defects in mitotic events. These findings emphasize the importance of CENP-C for mitotic progression and suggest that Vmw110 may be interfering with biochemical mechanisms which are relevant to both centromeres and ND10.

Results

Vmw110 interacts with centromeres in mitotic and interphase cells

This study arose from the observation that Hep2 cells undergoing mitosis following HSV-1 infection had a striking, reproducible and unusual distribution of Vmw110. While much of the protein was distributed diffusely throughout the cell, within the chromosomal region there were large numbers of small bright dots (Figure 1A). This localization was transient, since it was observed readily at early times of infection (2–4 h) but, by later times, Vmw110 staining was diffuse and excluded from the chromosomes (Figure 1B). Simultaneous staining for Vmw110, DNA and centromeres showed that the Vmw110 accumulations lay adjacent to and overlapping the centromeres (Figure 2A), a distribution that could be seen clearly on single chromosomes at high magnification (Figure 2B).

As described above, at the earliest stages of infection of interphase cells, Vmw110 migrates to ND10 and co-localizes with PML and the other constituent proteins. Detailed examination of infected cells revealed that in many of them, in addition to its localization at ND10, Vmw110 was also present in a number of smaller dots which did not appear to contain any PML (Figure 2D). Triple staining for PML, Vmw110 and centromeres clearly showed that many of these additional accumulations of Vmw110 were associated with centromeres, and in these cells almost all Vmw110 accumulations could be assigned to either ND10 or centromeres (Figure 2E and F). In contrast, any apparent association of ND10 and centromeres was rare (Figure 2G). Staining with the anti-CENP-B antibody confirmed that some Vmw110 associates with centromeres in both mitotic (Figure 2C) and interphase cells (Figure 2H).

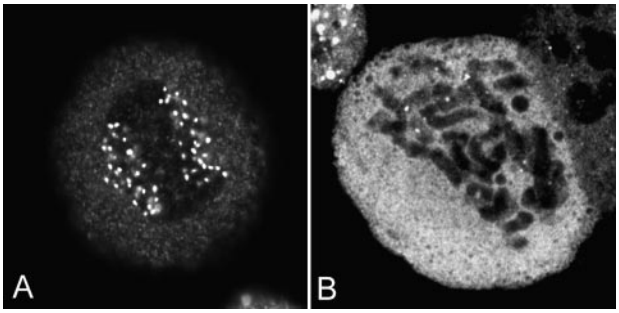


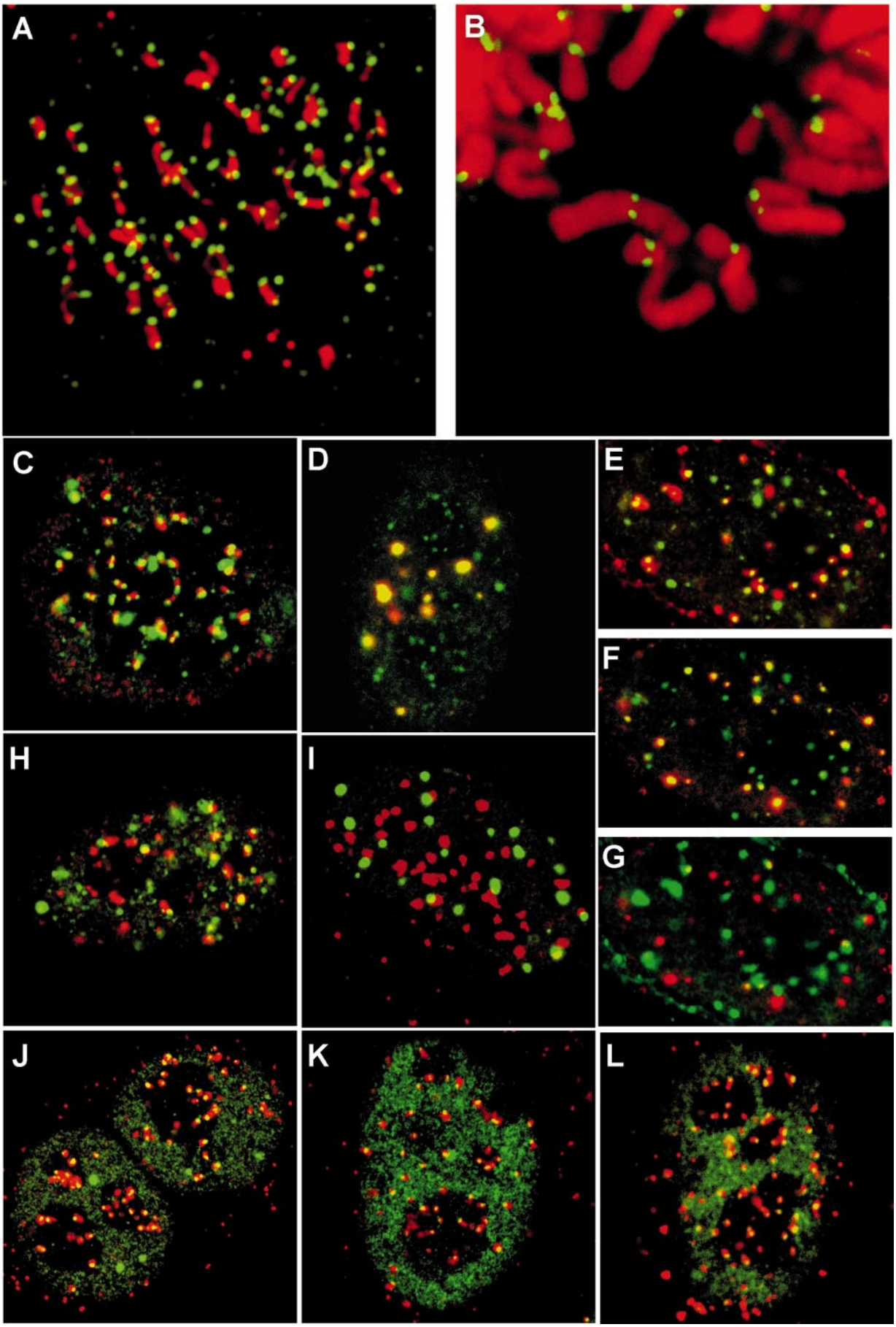
Fig. 1. Distribution of wild-type Vmw110 in infected mitotic cells. Mitotic cells stained for Vmw110 at 2 (**A**) and 6 h (**B**) after HSV-1 infection. In this and all other immunofluorescence figures, the magnifications vary between panels. Interphase Hep2 nuclei are on average ~10 μm across, while the mitotic cells have diameters of ~20 μm (see Figure 5H and K).

The RING finger of Vmw110 is required for its association with centromeres

To characterize further the interaction between Vmw110 and centromeres, we examined the effects of mutations in the Vmw110 RING finger domain and in sequences required for its binding to HAUSP and association with ND10. The properties of the various mutant proteins are summarized in Figure 3 and Table I.

Analysis of these mutants revealed a difference in the requirements for the protein to localize to centromeres and ND10. With the notable exception of the RING finger mutant FXE, all of the mutant proteins exhibited local accumulations in interphase cells that were associated with centromeres. These results separate the sequence requirements for Vmw110 localization at centromeres and ND10 in interphase cells, since the RING finger is required for the former (Figure 2I), but not the latter (Everett and Maul, 1994). Typical results for mutants Δ592/646 and Δ680/719 are shown in Figure 2J and K; similar patterns were obtained with mutants M1, Δ594/633, Δ634/679 and Δ723/767 (data not shown). Cells with this phenotype infected with the Δ594/775 mutant were rarer, but clear examples could be found (Figure 2L). The results with mutants Δ594/775, Δ634/679 and Δ680/719 are particularly striking since these proteins do not associate with ND10 in Hep2 cells and previously have been reported as having a diffuse nuclear distribution. However, the higher resolution microscopy used in this study clearly shows that in many cells in the early stages of infection, the C-terminal deletion mutants have small punctate accumulations within an overall diffuse distribution.

Examination of mitotic cells at the early stages of infection showed that the HAUSP-binding negative mutant proteins M1 and Δ594/633 associated with mitotic centromeres, but again the RING finger mutant FXE did not (Figure 4A–C). Mutant Δ592/646 gave a pattern strikingly different from the slightly smaller deletion Δ594/633, with minimal localization at mitotic centromeres (Figure 4D). Deletion Δ634/679 resulted in Vmw110 being mostly in a diffuse distribution, but some cells with residual mutant Vmw110 at centromeres could be located (Figure 4G). Mutant Δ680/719 was always distributed diffusely and excluded from chromatin in mitotic cells (Figure 4F), while the extreme C-terminus of Vmw110 was not required for mitotic centromere association (mutant Δ723/767; Figure 4H). The importance of the C-terminal region of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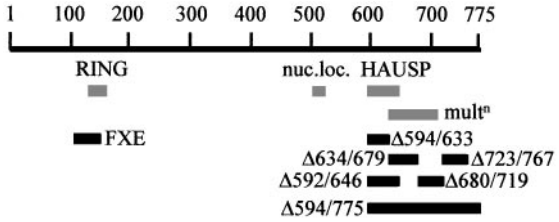

Fig. 3. A map showing the locations of the mutations in the 775 codon open reading frame of Vmw110 used in this study in relation to the RING finger, nuclear localization, HAUSP-binding and multimerization domains of the protein. Further details are shown in Table I.

Vmw110 for mitotic centromere localization was underlined by the diffuse distribution of the truncation mutant Δ594/775 (Figure 4E).

The results presented in Figures 2 and 4 are typical of most infected cells but, after examination of large numbers of cells infected with the mutant viruses in independent experiments, it became clear that it was not possible to ascribe a single, static phenotype for the distributions of the mutant proteins. For example, the location of the wild-type protein changed with time (Figure 1) and mutants M1 and Δ594/633 behaved in a similarly time-dependent manner. Mutant proteins Δ592/646 and Δ634/679 occasionally exhibited accumulations at mitotic centromeres, especially at early times of infection. However, mutant proteins FXE, Δ594/775 and Δ680/719 were never observed at centromeres in mitotic cells.

Vmw110 induces the proteasome-dependent degradation of CENP-C

Just as Vmw110 localization to ND10 is associated with the disruption of the subnuclear regions, so association of the protein with centromeres is associated with structural changes and functional disruption. Centromeres are large complex structures containing several proteins; we chose to examine in more detail the fate during infection of CENP-B and CENP-C, two major components of both interphase and mitotic centromeres (reviewed by Pluta *et al*., 1995). Staining with an anti-CENP-C serum revealed that CENP-C was not present in characteristic punctate dots in many infected cells, either in interphase (Figure 5B) or during mitosis (lower cell, Figure 5C; compare with the uninfected mitotic cell in Figure 5A). Figure 5B shows an uninfected interphase cell (lower left) stained for CENP-C (red) and two infected cells stained for Vmw110 (green). The lower infected cell has little if any remaining CENP-C, while the upper cell has a small number of foci stained for both antigens. Examination of a large number of cells indicated that the loss of CENP-C

Table I. Properties of the Vmw110 proteins expressed by the viruses used in the study

Virus	Vmw110 structure	Comments	ND10 localization	HAUSP binding
17+	1–775	Wild-type	+	+
*dl*1403	1–105	null mutant	–	–
FXE	1–105::150–775	RING finger Δ	+	+
Δ594/775	1–593	C-terminal truncation	–	–
Δ594/633	1–593::634–775		+	–
Δ592/646	1–591::647–775		+	–
Δ634/679	1–633::680–775		–	+
Δ680/719	1–679::720–775		–	+
Δ723/767	1–722::768–775		+	+
M1	R623L, K624I		+	–

Data assembled from Stow and Stow (1986), Everett (1989), Meredith *et al*. (1995) and Everett *et al*. (1999).

increased with time of infection and/or the amount of Vmw110 expression. Initially, significant co-localization of CENP-C and Vmw110 could be observed but, progressively, the number and intensity of CENP-C foci decreased until few or none remained. A similar situation was observed in mitotic cells, with transient co-localization of CENP-C and Vmw110 at centromeres (data not shown). It is important to note that Vmw110 did not cause a complete disintegration of centromeres since they clearly could be stained with autoimmune and anti-CENP-B sera (Figure 2), even in the absence of detectable CENP-C (data not shown).

This effect on CENP-C was investigated in cells infected with viruses expressing the Vmw110 mutant proteins. As predicted, the RING finger mutant FXE, which did not localize to centromeres, failed to induce the loss of CENP-C from either mitotic or interphase cells (Figure 5D and E). The C-terminal deletion mutants Δ680–719 (not shown) and Δ594/775 (Figure 5G) induced the loss of CENP-C from all cells; this result is particularly surprising as neither of these mutant proteins localized to mitotic centromeres. Although mutant M1 also induced the loss of CENP-C from centromeres in both mitotic and interphase cells, this effect was clearly less rapid than with wild-type virus since infected cells retaining CENP-C could be found much more easily. A typical result is shown in Figure 5F, with three infected cells expressing variable amounts of the mutant Vmw110 protein, all of which retain significant amounts of CENP-C. However, eventually cells infected with this mutant virus lost CENP-C staining (not shown). The slower loss of CENP-C induced by mutant M1 correlates with its reduced ability to induce the degradation of PML isoforms and disrupt ND10 in some cell types (Everett *et al*., 1998a), and it is

Fig. 2. Association of wild-type and mutant forms of Vmw110 with mitotic kinetochores and interphase centromeres 2 h after infection. (**A**) A mitotic cell infected with wild-type virus and stained for kinetochores with autoimmune serum 693 (red) and with mAb 11060 for Vmw110. (**B**) A high magnification view of individual chromosomes (red) with Vmw110 (green) localized at the kinetochore. (**C**) Vmw110 (green) localizing close to mitotic kinetochores stained for CENP-B (red). (**D**) An infected interphase cell stained for Vmw110 (green) and PML in ND10 (red), showing a high degree of co-localization of the two proteins, except at numerous smaller discrete accumulations of Vmw110. (E–G) Views of a similar cell, triple labelled for centromeres with autoimmune serum 693, PML and Vmw110. To simplify the interpretation, only two of the labels are shown in each panel: (**E**) centromeres (red) and Vmw110 (green); (**F**) PML (red) and Vmw110 (green); and (**G**) centromeres (green) and PML (red). Note that almost all of the Vmw110 dots in (E) and (F) can be accounted for as co-localizing either with PML (F) or centromeres (E), whereas centromeres and PML rarely co-localize (G). (**H**) An interphase cell with a proportion of Vmw110 (green) frequently juxtaposed or co-localizing with CENP-B (red). (**I**) An interphase cell infected with the FXE RING finger mutant (green) and its failure to associate with centromeres (autoimmune serum 693; red). (J–L) Interphase cells double stained for centromeres with autoimmune serum 693 (red) and mutant Vmw110 proteins expressed by viruses Δ592/646 (**J**), Δ680/719 (**K**) and Δ594/775 (**L**). The image in (A) was obtained using the DeltaVision system; all others are confocal images.

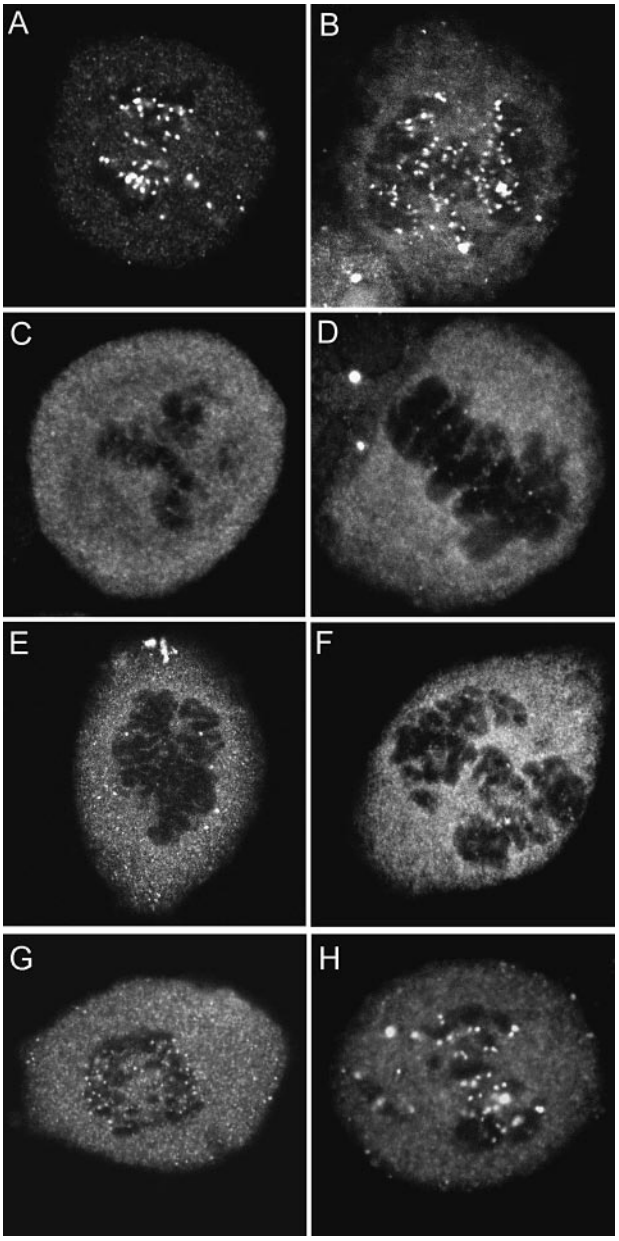

Fig. 4. Distribution of mutant Vmw110 proteins in mitotic cells 2 h after infection. (**A**) M1, (**B**) Δ594/633, (**C**) FXE, (**D**) Δ592/646, (**E**) Δ594/775, (**F**) Δ680/719, (**G**) Δ634/679 and (**H**) Δ723/767.

possible that these phenotypes correlate with the loss of the HAUSP-binding motif in this protein (Everett *et al*., 1999).

Since the Vmw110-dependent loss of CENP-C is reminiscent of the proteasome-dependent elimination of several PML isoforms in infected cells (Everett *et al*., 1998a), we investigated whether the proteasome inhibitor MG132 also stabilized CENP-C in the presence of Vmw110. Initial microscopy experiments showed that MG132 inhibited the Vmw110-induced loss of CENP-C (Figure 6). To distinguish between dispersal and degradation of the protein, samples of infected cell proteins were analysed by Western blotting. The results showed that the 140 kDa CENP-C band (Saitoh *et al*., 1992) was significantly reduced in virus-infected cultures, and this was dependent on the expression of functional Vmw110. Vmw110 null mutant virus *dl*1403 and the RING finger mutant FXE did not affect the amount of CENP-C and, most importantly, the reduction in CENP-C induced by wild-type virus was inhibited by MG132 (Figure 7). Consistent with the microscopy observations, the C-terminal deletion mutant Δ594–775 also induced the degradation of CENP-C, and this mutant reproducibly was more active than the wild-type in this assay (Figure 7).

Vmw110 alone is sufficient to induce the loss of CENP-C from centromeres

The experiments described above all involved virus infection, and while they clearly show that Vmw110 is essential for the effect of the virus on centromeres, it is possible that other virally induced factors are required. To test the effect of Vmw110 by itself, cells were transfected with plasmids expressing wild-type and mutant FXE and Δ594/775 Vmw110 proteins and stained for Vmw110 and CENP-C the following day. The results correlated very well with the virus infection experiments; cells expressing the wild-type and Δ594/775 proteins did not have any punctate CENP-C staining, whereas mutant FXE had little effect (Figure 8).

Vmw110-induced degradation of CENP-C results in mitotic disruption

CENP-C is an essential protein in mice (Kalitsis *et al*., 1998), and a previous study demonstrated that injection of anti-CENP-C antibodies led to reduction in the diameter of kinetochores, apparent disruption of microtubule binding, extended duration of mitosis and abnormal cytokinesis resulting in the production of micronucleated daughter cells (Tomkiel *et al*., 1994). To investigate whether Vmw110-induced degradation of CENP-C had similar consequences, Hep2 cells were synchronized by sequential thymidine and aphidicolin blocks, then infected with wild-type or mutant viruses at various times post-aphidicolin release. Uninfected cells progressed through mitosis with a peak frequency between 10 and 12 h after release; infections were initiated from 7 to 10 h post-release to create populations of cells in which Vmw110 had been expressed for a range of periods prior to the expected onset of mitosis. Cells were fixed at various times after infection, then stained for Vmw110 and DNA.

This experiment clearly showed that virus infection causes a profound disruption in mitotic events which can be attributed to the Vmw110-induced loss of CENP-C. Cells infected with wild-type virus were significantly delayed in mitosis, with the mitotic index rising as high as 40–50% (data not shown). Despite the presence of apparently normal microtubule spindles (data not shown), the majority of the infected mitotic cells exhibited extensive defects in chromosome alignment at a metaphase plate (Figure 5H). This phenotype showed a striking resemblance to cells in pseudoprometaphase induced by microinjection of anti-centromere antibodies (Bernat *et al*., 1990; Tomkiel *et al*., 1994). This was particularly marked if the cells had been infected for longer periods prior to the expected time of mitosis; if the cells were infected closer to mitosis, more apparently normal infected mitotic cells could be observed (see Figure 1). We also observed a number of infected cells undergoing cytokinesis or in early G_1, indicating that infection does not necessarily

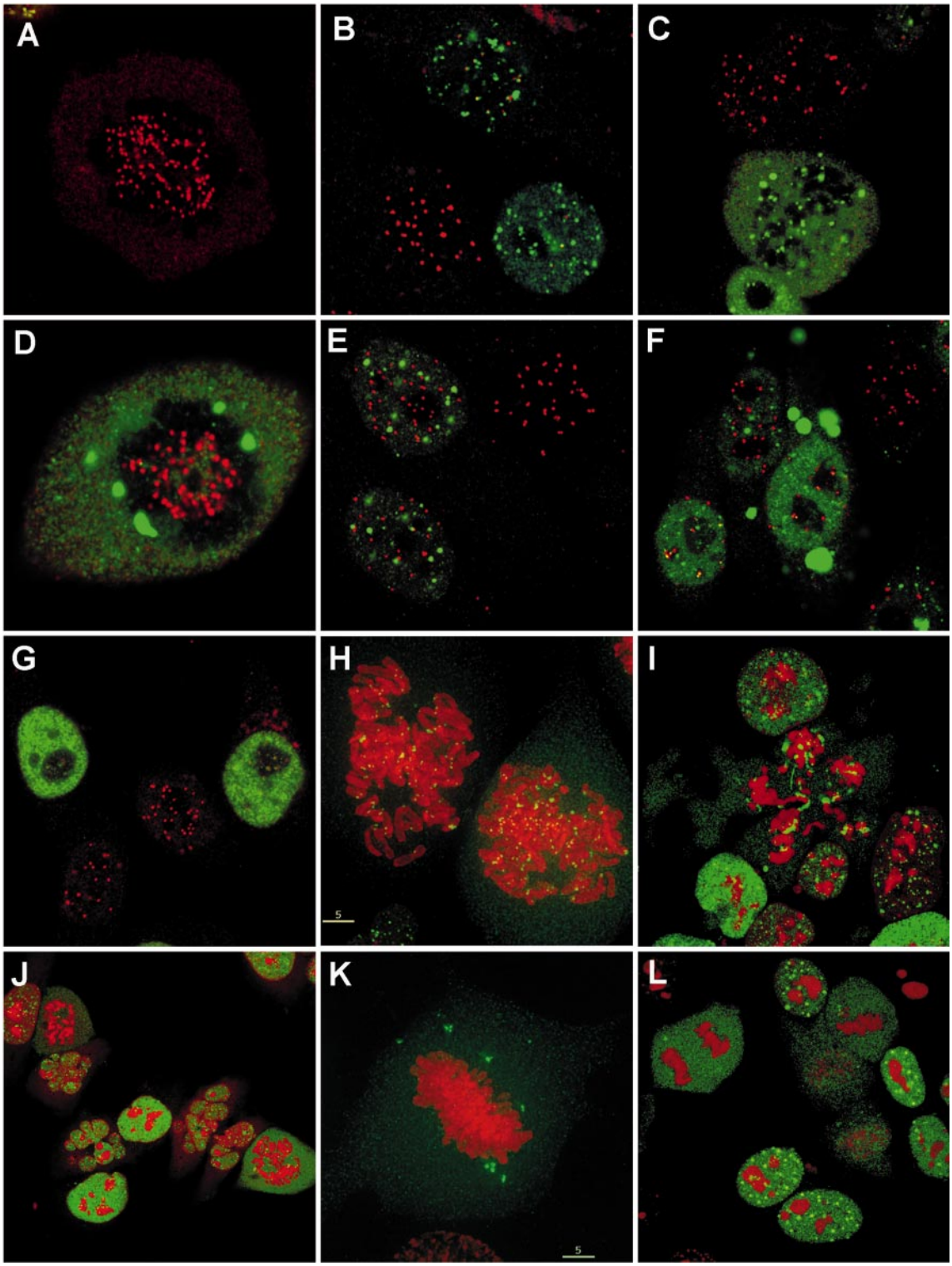

Fig. 5. The effect of virus infection on CENP-C and the consequence of its degradation in infected mitotic cells. (A–G) are stained for Vmw110 (green) and CENP-C (red). (**A**) An uninfected mitotic cell stained for CENP-C. (**B**) Interphase cells stained for Vmw110 and CENP-C. The lower left cell is uninfected, and the infected cell to its right has lost CENP-C staining. The upper cell has retained some CENP-C which co-localizes with a subset of the Vmw110 accumulations. (**C**) An infected mitotic cell which has no detectable CENP-C. (D and E) Cells infected with RING finger mutant FXE, showing retention of CENP-C in a mitotic cell (**D**) and in interphase cells (**E**), with no significant co-localization of the two proteins. (**F**) Three cells infected with HAUSP-binding mutant M1 with variable degrees of retention of CENP-C. (**G**) Loss of CENP-C from cells infected with C-terminal deletion mutant Δ594/775. (H–L) The consequences to mitotic cells of wild-type and FXE mutant virus infection, stained for Vmw110 (green) and DNA (red). (H and I) are typical views of wild-type infected cells showing cells in mitotic disarray (**H**) which results in abnormal cytokinesis (**I**) and daughter cells with many micronuclei (**J**). In contrast, FXE-infected cells predominantly go through normal metaphase (**K**), anaphase and cytokinesis (**L**). The images in (H) and (K) were obtained using the DeltaVision system, with the scale bar indicating 5 μm; all others are confocal images.

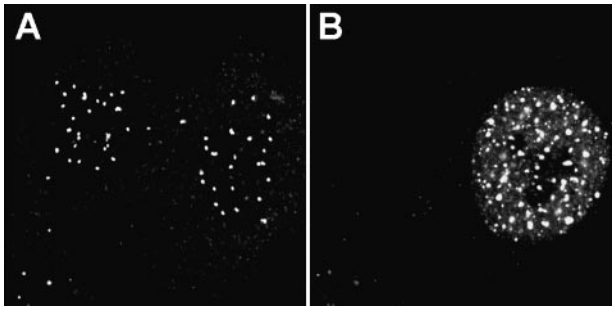

Fig. 6. The proteasome inhibitor MG132 stabilizes CENP-C in virus-infected cells. Hep2 cells were infected with HSV-1 in the presence of 2.5 μM MG132 and prepared for immunofluorescence 4 h later. A typical field of cells is shown, stained for CENP-C (**A**) and Vmw110 (**B**). The cell on the left is uninfected, while the cell on the right retains CENP-C despite abundant expression of Vmw110. Compare with Figure 5.

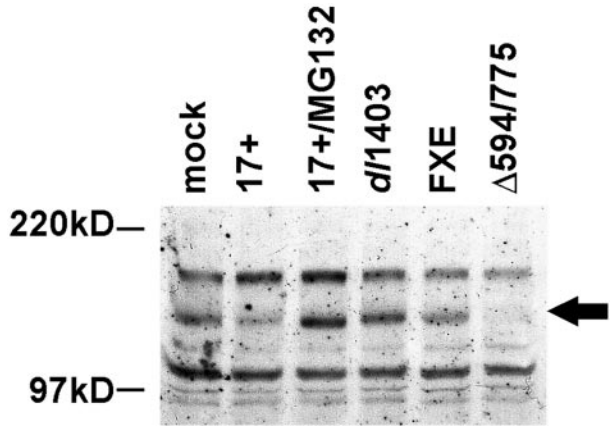

Fig. 7. Virus infection results in the Vmw110-induced and proteasome-dependent degradation of CENP-C. Hep2 cells were infected with wild-type (17+) and mutant viruses as shown at an m.o.i. of 5 p.f.u./cell and total cell proteins were harvested 8 h later. The proteins were analysed by Western blotting using anti-CENP-C serum r418. The arrow indicates the 140 kDa CENP-C band (identified by reprobing the blot with several different anti-CENP-C sera; data not shown), and the positions of the molecular weight markers are indicated. The effect of adding MG132 (2.5 μM) on the degradation of CENP-C induced by wild-type virus is shown in the third lane. Under these infection conditions, MG132 does not affect the expression of Vmw110 (Everett *et al*., 1998b).

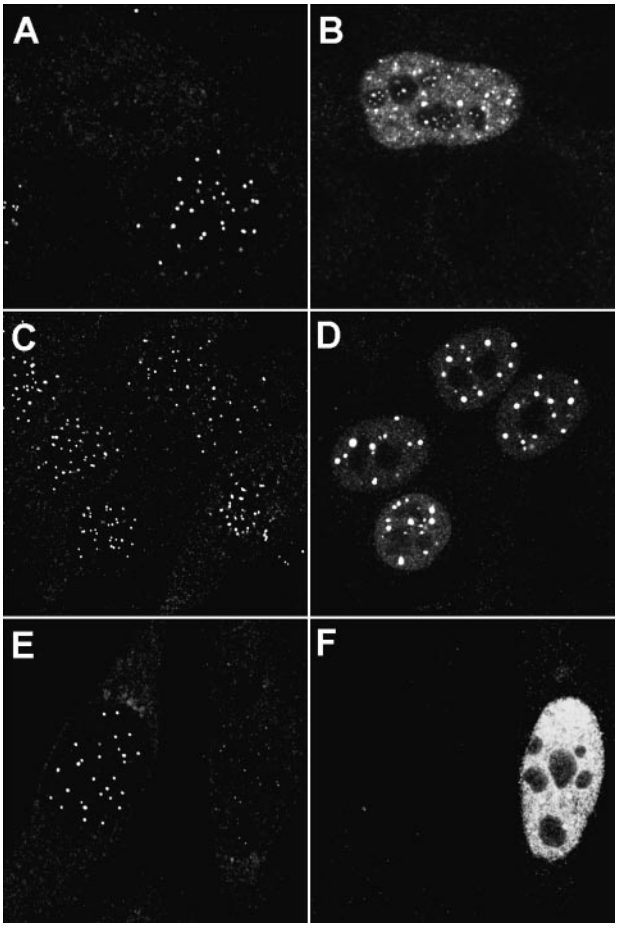

Fig. 8. Vmw110 is sufficient to induce the loss of CENP-C. Hep2 cells were lipofected with plasmids pCI110 (A and B), pCIFXE (C and D) and pCIΔ594/775 (E and F) and prepared for immunofluorescence 20 h later. The plasmids express wild-type Vmw110, the FXE RING finger deletion mutant and the Δ594/775 C-terminal truncation mutant, respectively. Paired panels show the same fields of cells, stained for CENP-C (A, C and E) or Vmw110 (B, D and F). (**A** and **B**) An untransfected cell (lower right) with typical CENP-C staining at centromeres, while the upper cell expressing wild-type Vmw110 has no detectable CENP-C. (**C** and **D**) A group of six cells, four of which are expressing the RING finger mutant Vmw110, FXE. These cells display readily detectable CENP-C. (**E** and **F**) A pair of cells either untransfected (left) or expressing the C-terminal deletion Vmw110, Δ594/775 (right). Expression of this protein again results in the loss of CENP-C staining.

cause a terminal arrest in mitosis. However, as in the case of cells injected with anti-centromere antibodies (Bernat *et al*., 1990; Tomkiel *et al*., 1994), most of these dividing cells were clearly abnormal. Cells undergoing cytokinesis with DNA bridges and malformed cleavage furrows were common (Figure 5I), as were daughter cells with many micronuclei (Figure 5J). These abnormal mitotic events were the result of the activities of Vmw110, since in contrast to wild-type virus infections, RING finger mutant virus FXE or deletion mutant *dl*1403 did not cause an accumulation of abnormal mitotic cells (data not shown) and instead the majority of FXE-infected dividing cells were predominantly in easily identifiable stages of metaphase (Figure 5K) or anaphase (Figure 5L). Furthermore, the FXE-infected cells could proceed through a normal mitosis to produce apparently normal early G_1 daughter cells (Figure 5L). It is perhaps surprising that virus-infected cells can progress successfully through mitosis, but these results show clearly that they can, providing functional Vmw110 is not expressed. The progression of virus infection is actually quite slow in Hep2 cells and, at the times used in these experiments, few cells show signs of abundant DNA replication and there are no discernible morphological effects on interphase cells.

Disruption of kinetochore structure induced by Vmw110

Previous studies cited above showed that the loss of CENP-C from kinetochores that occurs following microinjection of CENP-C antibodies resulted in disorganization of kinetochore structure, although chromosomes retained the ability to bind to microtubules. To characterize further the disruption of mitotic events caused by Vmw110, we

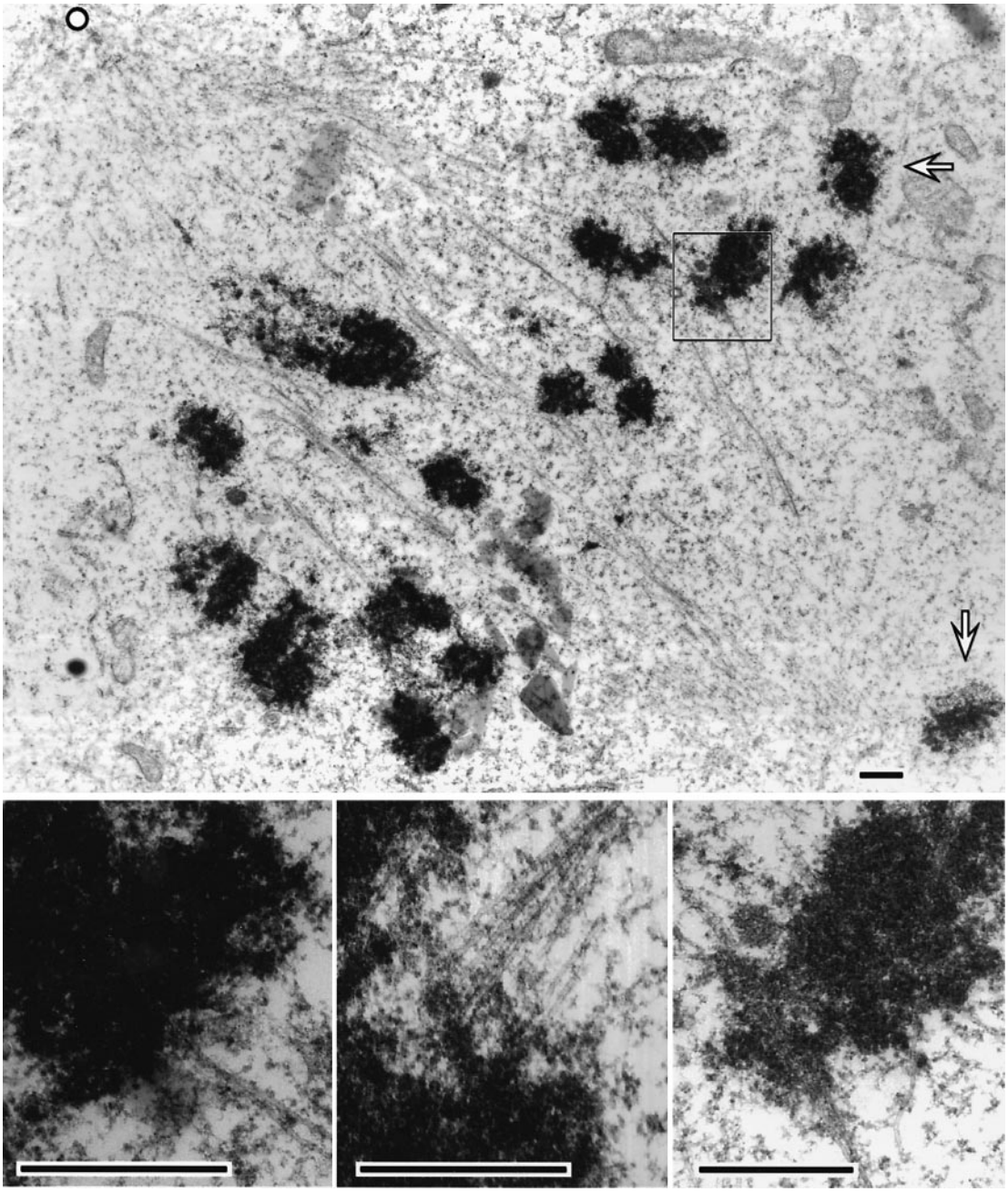

Fig. 9. Disruption of kinetochores in virally infected cells expressing wild-type Vmw110. Cells were released from an S phase arrest, infected with virus and harvested at various times thereafter. Upper panel: low magnification view of a mitotic cell with two maloriented chromosomes (arrows). Numerous other maloriented chromosomes could be seen in other serial sections from this cell. The boxed area is reproduced at higher magnification at the lower right. The circle at the upper left shows the approximate position of one spindle pole. Lower panels: higher magnification views of disrupted kinetochores from this cell. In all serial sections, microtubules were seen to enter the chromosome at regions that differed from bulk chromatin. These regions closely resembled disrupted kinetochores seen after anti-centromeric antibody injection (Bernat *et al*., 1991). The bar in all panels represents 0.5 μm.

studied the structure of kinetochores in infected cells by serial section electron microscopy; selected images are shown in Figure 9. With striking similarity to cells injected with anti-centromere antibodies, these cells contained relatively normal bipolar spindles but, as observed by light microscopy (Figure 5H), many of the chromosomes failed to align at a compact metaphase plate. Examination at higher magnification revealed that the chromosomes were associated with bundles of microtubules that appeared to correspond to kinetochore fibres, but these microtubules did not end in normal trilaminar kinetochore structures. Instead, they seemingly were attached to the chromosomes at amorphous structures, which often appeared to be being unravelled from the chromosome surface. Taken together with the previous results, we conclude that the Vmw110-induced loss of CENP-C from kinetochores, like the antibody-induced loss of CENP-C (Tomkiel *et al*., 1994), resulted in a profound disruption of kinetochore structure.

These results therefore confirm previous conclusions concerning the importance of CENP-C for the structure of the trilaminar kinetochore.

Discussion

This study demonstrates that HSV-1 regulatory protein Vmw110 associates with centromeres at the early stages of infection of both interphase and mitotic cells. Consequently, centromere protein CENP-C is degraded, kinetochore structure is disrupted and mitosis is stalled, delayed or abnormal. The parallels between the effects of Vmw110 on ND10 and centromeres suggest that related mechanisms may be involved in both processes and reveal a previously unsuspected connection between these seemingly separate nuclear structures.

The mechanism of Vmw110-induced loss of CENP-C

The requirement for the RING finger of Vmw110 for the loss of CENP-C, and the inhibition of this effect by MG132, precisely mirror the virus-induced loss or degradation of a number of high molecular weight PML isoforms (Everett *et al*., 1998a). The isoforms that are affected preferentially have gel mobilities very similar to SUMO-1-conjugated PML proteins produced in transfected cells (Sternsdorf *et al*., 1997b; Kamitani *et al*., 1998; Muller *et al*., 1998). A reasonable hypothesis is that the endogenous high molecular weight PML isoforms represent SUMO-1 conjugates, although the low amounts of PML in the cell and its insolubility have so far precluded formal proof. In addition, it has been shown that Vmw110 can induce the loss of a number of undefined SUMO-1-conjugated proteins (Everett *et al*., 1998a) and, therefore, a more general mechanism may be operating in which Vmw110 is targeting SUMO-1-modified proteins, or the SUMO-1 conjugation or deconjugation pathways.

Previous studies concerning the centromeric autoantigens suggested that these proteins might bear a common post-translational modification that is recognized by human autoantibodies (Earnshaw and Rothfield, 1985). The data presented here, together with the results of previous studies on the effect of Vmw110 on the stability of PML isoforms, suggest that CENP-C may be modified by SUMO-1 or a similar ubiquitin-like protein. Indeed, a variety of evidence has accumulated in recent years to suggest that centromere assembly may be regulated by epigenetic mechanisms, and it is tempting to speculate that SUMO-1 modification could be involved. For example, temperature-sensitive mutations in the budding yeast homologue of CENP-C, MIF-2 (Meluh and Koshland, 1995), can be suppressed by high level expression of Smt-3, the yeast homologue of SUMO-1 (Meluh and Koshland, 1995; DDBJ/EMBL/GenBank accession No. U27233). An intriguing speculation is that modification of CENP-C may in some way be involved in marking the active centromere. It will be important in future experiments to identify any SUMO-like modification of CENP-C, but this is likely to be complicated by the small amounts of CENP-C in the cell.

The cellular consequences of centromere modification by Vmw110

Loss of CENP-C mediated by Vmw110 has serious consequences for kinetochore structure and function, resulting in defects in chromosome alignment and an extended mitotic delay. Our results confirm the essential role of CENP-C in the kinetochore deduced from injection of anti-CENP-C antibodies (Tomkiel *et al*., 1994), disruption of the CENP-C gene (Kalitsis *et al*., 1998) and conditional disruption of CENP-C function in chicken DT40 cells (Fukagawa and Brown, 1997). If infection was initiated in late G_2, the mitotic delay imposed by Vmw110-mediated CENP-C destruction was only temporary, at least in those cells which undergo an abortive anaphase and produce highly aberrant offspring (Figure 5I and J). This resembles the phenotype seen following injection of anti-CENP-C antibodies, and is reminiscent of the defects seen in budding yeast NDC10 mutants at non-permissive temperature. Thus elimination of CENP-C after a critical time in the cell cycle may only temporarily activate a metaphase checkpoint, allowing some cells to adapt and resume mitotic progression. It will be interesting in future studies to stain cells expressing Vmw110 for various kinetochore-associated checkpoint components, such as hBub1, hBubR1 and hMad2, to see if these markers reveal abnormalities in kinetochores lacking CENP-C.

The destruction of CENP-C and subsequent mitotic disruption induced by Vmw110 explains several previous observations: (i) inclusion of a plasmid expressing Vmw110 drastically reduced the number of colonies obtained in biochemical transformation experiments (Johnson *et al*., 1994); (ii) there are no published examples of cell lines which express Vmw110 constitutively and, despite several attempts by various methods, we have been unable to achieve this; (iii) infection with replication-defective viruses which express Vmw110, but not other essential viral IE regulatory proteins, is cytotoxic despite the absence of lytic replication in the infected cells (Wu *et al*., 1996; Samaniego *et al*., 1998). Therefore, while the effect of Vmw110 on PML and other cellular proteins may itself have an effect on cell viability, it is certain that the Vmw110-induced loss of CENP-C will lead to failure of a dividing cell population to propagate. It is an interesting speculation that this property of Vmw110 has contributed to the biology of HSV-1 latency, which occurs in non-dividing cells that repress the transcription of viral protein-encoding genes. However, it is now clear that the design of vectors for gene therapy based on HSV-1 must take account of the effect that any expressed Vmw110 would have on the target cell population.

The nature of the Vmw110–centromere association

The localization of Vmw110 at centromeres must be occurring through specific sequences of Vmw110 interacting with one or more centromere components. The only mutant Vmw110 protein which failed to associate with either interphase or mitotic centromeres, or induce the degradation of CENP-C, was the RING finger deletion FXE. Therefore, it is possible that the RING finger makes specific contacts with proteins within the centromere. However, analysis of the behaviour of the other mutants is not consistent with a simple model. Mutations in the C-terminal region of the protein encompassing the HAUSP-binding, self-multimerization and ND10 localization domains of Vmw110 gave complex results which varied both with time of infection and whether interphase or mitotic centromeres were being examined (summarized

Table II. Summary of the centromere association of mutant Vmw110 proteins

Virus	Vmw110 structure	Interphase centromeres	Mitotic centromeres	Loss of CENP-C	ND10 disruption
17+	1–775	++	++	++	++
FXE	1–105::150–775	–	–	–	–
Δ594/775	1–593	+/–	–	++	–
Δ594/633	1–593::634–775	++	++	+	+
Δ592/646	1–591::647–775	++	+/–	+	+
Δ634/679	1–633::680–775	+	+/–	++	–
Δ680/719	1–679::720–775	+	–	++	–
Δ723/767	1–722::768–775	+	++	++	++
M1	R623L, K624I	++	++	+	+

The relative abilities of the mutant proteins to associate with interphase and mitotic centromeres, to induce the loss of CENP-C (as visualized by microscopy, and confirmed by Western blotting) and to disrupt ND10 (Everett and Maul, 1994; Meredith *et al*., 1995; Everett *et al*., 1999) are indicated in comparison with wild-type Vmw110. Loss of CENP-C and disruption of ND10 vary with time of infection and cell type, and are not accurately quantifiable. A single + in these columns indicates that these mutants were positive, but reproducibly less active than wild-type in Hep2 cells 4 h post-infection, while – indicates lack of activity at this time. Localization at interphase and mitotic centromeres again varied with time and was most obvious at early times of infection (see text): ++ indicates similar to wild-type; + indicates association in fewer cells, frequently partly masked by a bright diffuse background; +/– indicates association in rare cells; – indicates never observed.

in Table II). For example, accumulations of mutant Δ680/719 could be found readily at interphase centromeres early in infection, but they were never observed at mitotic centromeres. The differences in localization could be influenced by the biochemical effects of the Vmw110 proteins; over a number of repeated experiments, the C-terminal truncation mutant Δ594/775 was probably the most active in eliminating CENP-C (even more so than the wild-type; Figure 7) and it was also the most difficult to observe in association with interphase centromeres.

The identity of the centromeric Vmw110 binding partner is also of interest. Since the localization of Vmw110 at centromeres is transitory (Figure 1), it is possible that the partner is CENP-C itself. However, we note that Vmw110 can remain at centromeres after CENP-C has been lost, the degree of Vmw110 localization at centromeres varies from cell to cell (perhaps with cell cycle status) and, even within individual interphase cells, the proportion of centromeres containing detectable Vmw110 is variable. These differences could reflect changes in composition and structure of the centromere during interphase and mitosis. There is a precedent for such changes since hDaxx (a protein implicated in the modulation of the cell death response) can localize with a subset of centromeres, but only during interphase (Pluta *et al*., 1998). A complete understanding of the interaction of Vmw110 with centromeres will require further information on its effects on centromere structure and components.

The possible relationship of centromere modification to the biological activities of Vmw110 during the viral life cycle

One vexing question raised by the present results is why a virus such as HSV-1 would degrade a kinetochore component and thereby disable the mitotic process in infected cells. This is particularly puzzling given that active HSV-1 infections result in cell death. We suggest three possible explanations for the phenomena described here:

A potential anti-premature apoptosis mechanism. We have shown recently that CENP-C can interact *in vivo* with hDaxx, a human homologue of a murine protein originally identified as a mediator of the Fas/CD95 apoptotic pathway (Yang *et al*., 1997; Pluta *et al*., 1998). In addition, it has been shown that inactivation of CENP-C in the context of a CENP-C–oestrogen receptor fusion protein leads to terminal mitotic arrest followed by apoptosis (Fukagawa and Brown, 1997). Therefore, the destruction of CENP-C may be important for the virus to prevent the cell from aborting the viral infection by inducing an apoptotic response.

A consequence of a common pathway. It is becoming increasingly clear that Vmw110 affects the stability of several cellular proteins. In addition to CENP-C, examples of proteins that become less stable include selected isoforms of PML and another major ND10 component, sp100 (Everett *et al*., 1998a; R.Everett, T.Sternsdorf and H.Will, unpublished data), and the catalytic subunit of DNA-PK (Lees-Miller *et al*., 1996; Parkinson *et al*., 1999). The primary target of Vmw110 could be modified proteins such as PML and sp100 whose destruction is important for the course of the lytic infection, and CENP-C could simply be an 'innocent bystander' that is destroyed because it carries a SUMO-like modification.

Vmw110 may inactivate a silencing or repression mechanism which is in some way connected to centromeric heterochromatin. Since the proteasome inhibitor MG132 not only inhibits the Vmw110-induced degradation of all of the examples cited above, but also inhibits the ability of Vmw110 to stimulate the onset of virus infection and reactivation from quiescence (Everett *et al*., 1998b), it is highly likely that the biological activities of Vmw110 during the virus life cycle are a consequence of its effects on protein stability. We have recently put forward a model which suggests that Vmw110 inhibits the cell-mediated repression of the whole viral genome which preferentially occurs in its absence (Everett *et al*., 1998b). We proposed that as yet undefined host repression proteins target the incoming viral genome and compete with transcriptional activation induced by VP16 and other virally encoded *trans*-acting factors. If Vmw110 were to induce the degradation of a key component of the repression mechanism, the probability of transcriptional activation prevailing

would be increased. Recent evidence has revealed some intriguing links between this model and the effects of Vmw110 at ND10 and centromeres. It has been shown that the heterochromatin-binding protein HP1 interacts with the ND10 component sp100, thereby suggesting for the first time a link between ND10 and the chromatin compartment (Lehming *et al*., 1998; Seeler *et al*., 1998). The human HP1 cDNA was first cloned by screening using autoimmune sera which recognize a group of proteins that frequently are recognized also by sera with anti-centromere antibodies (Saunders *et al*., 1993). The related *Drosophila* HP1 protein associates with heterochromatin, including that at centromeres, and has been shown to be an enhancer of position effect variegation, a characteristic of proteins that promote transcriptional silencing (James *et al*., 1989; Eissenberg *et al*., 1990, 1992). Therefore, it appears that at least one component of a potential host silencing or repression system is present both at ND10 and centromeres; indeed, recent results indicate that accumulations of HP1 can be found associated with both of these structures (R.D.Everett and W.C.Earnshaw, in preparation). Therefore, the effects of Vmw110 on PML, sp100 and CENP-C may reflect the presence of factors at both ND10 and centromeres involved in the regulation of the viral genome. We are currently investigating this possibility.

Materials and methods

Viruses, cells and plasmids

HSV-1 strain 17+ was the wild-type virus used in these studies. Vmw110 mutant viruses *dl*1403, FXE, Δ594/633 (previously known as D12), Δ634/679 (previously known as D13), Δ680/719 (previously known as D14), Δ723/767 (previously known as D15) and Δ594/775 (previously known as E52X) have been described previously (Stow and Stow, 1986; Everett, 1989; Meredith *et al*., 1995). Deletion mutant virus Δ592/646 (previously known as A78) and substitution mutant virus M1 express Vmw110 proteins that do not bind to HAUSP (Everett *et al*., 1999; for details see Table I and Figure 3). All viruses were grown and titrated in baby hamster kidney (BHK) cells propagated in Glasgow modified Eagle's medium containing 100 U/ml penicillin and 100 μg/ml streptomycin, and supplemented with 10% newborn calf serum (NCS) and 10% tryptose phosphate broth. Hep2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics as above. Plasmids pCI110, pCIFXE and pCIΔ592/775 (formerly known as pCIE52X) have been described previously (Everett *et al*., 1999).

Synchronization of cells

Monolayers of synchronized cells were produced by sequential thymidine and aphidicolin blocking steps. Cells were seeded at a density of 1×10^5 per 35 mm dish containing four coverslips. The following day, medium containing 2 mM thymidine was substituted and, 12 h later, the cells were washed twice and medium containing 0.025 mM thymidine and 0.025 mM deoxycytidine added. A further 12 h later, the cells were re-fed with medium containing 2.5 μg/ml aphidicolin and, after another 14 h, the cells were washed twice and re-fed with normal medium. Virus infections were initiated 7–10 h after aphidicolin release as appropriate, with the peak period of mitosis occurring 10–12 h after release from the aphidicolin block.

Antibodies

Anti-Vmw110 mAb 11060 and the anti-PML rabbit serum r8 have been described previously (Everett *et al*., 1993; Boddy *et al*., 1996). Human autoimmune serum 693, which recognizes centromeric proteins, was a gift from Professor Gerd Maul. Rabbit serum rL was generated using recombinant CENP-B as immunogen, and sera r418 and r554 recognize CENP-C (Cooke *et al*., 1990; Tomkiel *et al*., 1994). The secondary antibodies used were tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG, fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG, TRITC-conjugated goat anti-human IgG (Sigma) and Cy5-conjugated goat anti-mouse, anti-rabbit and anti-human IgG (Amersham).

Immunofluorescence

Hep2 cells were seeded onto coverslips in Linbro wells at a density of 5×10^4 cells per well 1 day prior to infection. The cells were infected at a multiplicity of infection (m.o.i.) of 10 plaque-forming units (p.f.u.) per cell and fixed with formaldehyde [5% (v/v) in phosphate-buffered saline (PBS) containing 2% sucrose] at various times after infection as noted in the text. After fixation, the cells were permeabilized with 0.5% NP-40 in PBS with 10% sucrose. The primary antibodies were diluted in PBS containing 1% NCS. Antibodies were used at dilutions as follows: anti-Vmw110 mAb 11060, 1/1000; anti-PML rabbit serum r8, 1/1000; 693 autoimmune serum, 1/200; anti-CENP-B serum rL, 1/1000; and anti-CENP-C rabbit serum r554, 1/300. After incubation at room temperature for 1 h, the coverslips were washed at least six times then treated with secondary antibodies. The Sigma reagents were used at 1/100–1/200, while the Amersham antibodies were used at 1/200–1/500. After a further 60 min incubation, the coverslips were again washed at least six times and mounted using Citifluor. Where appropriate, DNA was stained using propidium iodide at a concentration of 1 μg/ml for 5 min after the secondary antibody incubations.

Light microscopy

Most stained cell samples were examined using a Zeiss LSM 510 confocal microscope with three lasers giving excitation lines at 633, 543 and 488 nm. The data from the channels were collected either simultaneously or, when necessary, separately, using the narrow band pass filter settings built into the instrument. Double labelling experiments usually employed a combination of FITC and Cy5 dyes to eliminate channel overlap effects. Triple labelling utilized dye combinations and laser intensities which, given the intrinsic strengths of the signals due to the characteristics of the primary antibody and the abundance of its cognate protein, were found to produce undetectable overlap problems. Specimens were tested for overlap by turning off individual laser lines while continuously scanning the three channels. Data was collected with 8-fold averaging at a resolution of 1024×1024 pixels using optical slices of between 0.5 and 1 μm. The microscope was a Zeiss Axioplan utilizing a 63× oil immersion objective lens, NA 1.4. Data sets were processed using the LSM 510 software, then exported for preparation for printing using Photoshop. Where indicated, three-dimensional data sets of selected mitotic cells were collected using a DeltaVision microscope (Applied Precision), based on an Olympus IX-70 inverted microscope with a Chroma Technology multiple bandpass filter set and a Photometrics PXL cooled CCD camera. Data sets were deconvolved, projected onto a single plane and the contrast was adjusted prior to export as TIFF files to Adobe Photoshop.

Western blotting

Hep2 cells were seeded at 1×10^5 cells per well in 24-well Linbro multi-well plates and infected the following day with viruses at an m.o.i. of 5 p.f.u./cell. The cells were washed with PBS and harvested into SDS–gel loading buffer 8 h after virus adsorption. Total cell proteins from 5×10^4 cells were loaded onto 7.5% SDS–polyacrylamide gels set in a Bio-Rad MiniProtean II kit, then electroblotted to nitrocellulose membranes using the compatible Bio-Rad equipment. The membranes were blocked at 4°C overnight using 5% dried milk in PBS containing 0.1% Tween-20 (PBST), then incubated with anti-CENP-C rabbit serum r418 at a dilution of 1/1000 in PBST/5% dried milk. After 4 h, the membrane was washed extensively, then incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, 1/50 000 in PBST/2% dried milk). Bound antibody was detected using the Amersham ECL method.

Electron microscopy

Synchronized Hep2 cells were prepared as described above (except that Aclar coverslips were used), then infected with wild-type 17+ virus at an m.o.i. of 10 p.f.u./cell. Infections were initiated 8 h post-aphidicolin release; 4 h later the cells were washed with PHEM (60 mM PIPES pH 6.9, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pre-warmed to 37°C), permeabilized for 1 min with PHEM plus 0.1% Triton, fixed for 20 min in 2% glutaraldehyde in PHEM, rinsed three times in PHEM, then post-fixed with 1% OsO_4 in PHEM for 30 min. Pre-staining was performed with 2% uranyl acetate in 30% ethanol for 1 h, then the nuclei were dehydrated in ethanol and transferred through a propylene oxide series to Spurrs resin. Gold sections were cut with a Reichert

UCT microtome, placed on 200 mesh copper grids, and observed using a Philips CM120 Biotwin electron microscope. Negatives were printed to photographic paper. The resultant images were scanned and assembled into a figure using Adobe Photoshop.

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