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A dynamic connection between centromeres and ND10 proteins

Roger D. Everett[1,*], William C. Earnshaw[2], Ann F. Pluta[3], Thomas Sternsdorf[4], Alexandra M. Ainsztein[5], Mar Carmena[2], Sandrine Ruchaud[2], Wei-Li Hsu[1] and Anne Orr[1]

[1]MRC Virology Unit, Church Street, Glasgow G11 5JR, Scotland UK
[2]Institute of Cell and Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR, Scotland, UK
[3]Department of Molecular Biology and Biophysics, University of Maryland Biotechnology Institute, 725 West Lombard Street, Baltimore, Maryland 21201 USA
[4]Heinrich-Pette-Institut, Martinistrasse 52, 20251 Hamburg, Germany
[5]Laboratory of Molecular Embryology NIH/NICHD, 18 Library Drive, Bethesda, MD 20892 USA
*Author for correspondence (e-mail: r.everett@vir.gla.ac.uk)

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SUMMARY

ND10, otherwise known as nuclear dots, PML nuclear bodies or PODs, are punctate foci in interphase nuclei that contain several cellular proteins. The functions of ND10 have not been well defined, but they are sensitive to external stimuli such as stress and virus infection, and they are disrupted in malignant promyelocytic leukaemia cells. Herpes simplex virus type 1 regulatory protein Vmw110 induces the proteasome-dependent degradation of ND10 component proteins PML and Sp100, particularly the species of these proteins which are covalently conjugated to the ubiquitin-like protein SUMO-1. We have recently reported that Vmw110 also induces the degradation of centromere protein CENP-C with consequent disruption of centromere structure. These observations led us to examine whether there were hitherto undetected connections between ND10 and centromeres. In this paper we report that hDaxx and HP1 (which have been shown to interact with CENP-C and Sp100, respectively) are present in a proportion of both ND10 and interphase centromeres. Furthermore, the proteasome inhibitor MG132 induced an association between centromeres and ND10 proteins PML and Sp100 in a significant number of cells in the G_2 phase of the cell cycle. These results imply that there is a dynamic, cell cycle regulated connection between centromeres and ND10 proteins which can be stabilised by inhibition of proteasome-mediated proteolysis.

Key words: ND10, PML nuclear domain, Centromere, Proteasome inhibitor

INTRODUCTION

The mammalian cell nucleus contains a large number of structural entities which are engaged in the various activities of transcription, RNA processing, DNA replication and chromosome dynamics (reviewed by de Jong et al., 1996; Lamond and Earnshaw, 1998). The composition and functions of some of these structures (such as the nucleolus and centromeres) are fairly well understood, but others are less well defined. One of the best known, but least understood nuclear sub-structure is the domain variously known as ND10, nuclear dots, PML nuclear bodies or promyelocytic oncogenic domains (PODs). Depending on cell type, there are usually between 5 and 20 punctate ND10 structures of about 0.5 μm diameter in the nucleus, each containing several proteins of which the most studied are PML and Sp100 (reviewed by Sternsdorf et al., 1997a). Interest in PML arose because many cases of promyelocytic leukaemia are caused by a chromosomal translocation which results in the expression of a fusion protein linking the normal PML protein to the retinoic acid receptor α (RARα) (for references, see Sternsdorf et al., 1997a). Recent work has implicated the normal PML protein in growth suppression (Mu et al., 1994), haemopoietic differentiation (Wang et al., 1998a), transcription (Alcalay et al., 1998; Vallian et al. 1998), regulation of MHC class I antigen presentation (Zheng et al., 1998) and regulation of apoptosis (Wang et al., 1998b). Less is known about Sp100, but two recent papers have shown that it interacts with the heterochromatin-associated protein HP1 and that it may have a role in repression of gene expression (Seeler et al., 1998; Lehming et al., 1998). Both PML and Sp100 are modified by covalent linkage to the ubiquitin-like protein SUMO-1 (Sternsdorf et al., 1997b; Muller et al., 1998; Kamitani et al., 1998a, b; Duprez et al., 1999). Other components of ND10 include the little characterised NDP55 (Maul et al., 1995) and there are a number of other proteins which can be found in a subset of ND10, including HAUSP (a ubiquitin-specific protease) (Everett et al., 1997) and the PML-related ret finger protein RPF (Cao et al., 1998).

The dynamic nature of ND10 is well illustrated by their response to virus infection. In particular, herpes simplex virus type 1 (HSV-1) infection leads to ND10 disruption within a few hours (for references, see Everett et al., 1998a). It is now clear that this disruption is caused by the viral immediate-early regulatory protein Vmw110, which first accumulates at ND10,

then induces the degradation of the SUMO-1 modified forms of PML and Sp100 in a proteasome-dependent manner (Everett et al., 1998a; Chelbi-Alix and de The, 1999). Vmw110 also associates with centromeres and induces the proteasome-dependent degradation of centromeric protein CENP-C (Everett et al., 1999). The parallels between the effects of Vmw110 on ND10 and centromeres, and the observation that Vmw110 could associate with both structures, led us to explore whether there was any previously undetected connection between these seemingly separate nuclear structures in uninfected cells.

We have taken several approaches to investigate this possibility. Firstly, we examined in more detail the distribution of hDaxx, a CENP-C interacting protein which had been detected in centromeres and other uncharacterised intranuclear foci (Pluta et al., 1998), and found that it is a component of both ND10 and centromeres. Secondly, we extended previous observations on the presence of heterochromatin protein HP1 in both ND10 and centromeres. Thirdly, we examined the relative distributions of ND10 proteins and centromeres in cells after a variety of treatments and found a surprising cell cycle dependent association of PML and Sp100 with centromeres after treatment with the proteasome inhibitor MG132. These results suggest that ND10 proteins have a previously unsuspected, dynamic relationship with interphase centromeres and provide further support for recent work which suggests a link between ND10 and the chromatin compartment.

MATERIALS AND METHODS

Cells

Human epithelial Hep2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. SUMO13 cells constitutively expressing a myc-tagged version of SUMO-1 were isolated after transfection of plasmid pCIPIC1 (Everett et al., 1998a) into HeLa cells, then screening G418 resistant colonies for expression of the tagged SUMO-1. In all the lines isolated, expression of tagged SUMO-1 varied between individual cells, but re-cloning allowed the maintenance of stocks in which up to 10% of the cells expressed readily detectable amounts of tagged SUMO-1. SUMO13 cells were maintained in DMEM supplemented with 2.5% foetal calf serum, 2.5% new born calf serum, 400 μg/ml G418, 100 units/ml penicillin and 100 μg/ml streptomycin.

Monolayers of synchronised cells were produced by sequential thymidine and aphidicolin blocking steps. Cells were seeded at a density of 1×10^5 per 35mm dish containing 4 coverslips. The following day, medium containing 2 mM thymidine was substituted, and 12 hours later the cells were washed twice and medium containing 0.025 mM thymidine and 0.025 mM deoxycytidine added. A further 12 hours later the cells were re-fed with medium containing 2.5 μg/ml aphidicolin, and after another 12 hours the cells were washed twice and re-fed with normal medium. The success of the synchronisation procedure was monitored visually, by analysis of parallel or similarly treated samples by FACS, and by western blotting for cyclin B.

NB4 cells derived from a patient with acute promyelocytic leukaemia were a gift from Dr M. Lanotte at INSERM U-496, Paris (Lanotte et al., 1991). They were grown in RPMI 1640 medium supplemented with 10% FCS and prepared for immunofluorescence by centrifugation onto coverslips prior to fixation and immunostaining as described below.

Treatment with the proteasome inhibitor MG132 (Calbiochem; 5 μM final concentration) and human interferon α (1000 units/ml) was as detailed in the text and figure legends. The caspase inhibitor zVAD was used at 100 μM for 6 hours.

Antibodies

The following antibodies were used at the indicated dilutions for immunofluorescence experiments: anti-hDaxx rabbit serum r1866 (1/1000; Pluta et al., 1998); anti-PML rabbit serum r8 (1/1000; Boddy et al., 1996); anti-PML monoclonal antibody 5E10 (1/10; Stuurman et al, 1992); anti-Sp100 rabbit serum spGH (1/1000; Sternsdorf et al., 1995); anti-Sp100 rat serum sp26 (1/1000; Grötzinger et al., 1996); anti-CENP-C rabbit serum r554 (1/500; Saitoh et al., 1992); human serum GS with anti-centromere antibodies (1/20,000). Anti-$HP1^{Hs\alpha}$ polyclonal mouse serum was produced by the following procedure: $HP1^{Hs\alpha}$ fused to glutathione S-transferase was expressed in *E. coli* and purified on glutathione columns. The GST:$HP1^{Hs\alpha}$ was released from the column by boiling in SDS sample buffer, resolved in SDS-PAGE, stained with aqueous Coomassie blue, and the relevant band excised. This band was frozen under liquid nitrogen, ground to a fine powder, resuspended in PBS, and used for polyclonal antibody production in mice by a modification of the method of Ou et al. (1993). Female Balb/c mice were immunised with 10-20 μg of protein per injection (Scottish Antibody Production Unit, Carluke). Following three injections, a test bleed was assayed for anti-$Hp1^{Hs\alpha}$ by immunoblotting and indirect immunofluorescence. Mice that were producing antibody were then 'primed' by injection with 0.2 ml of Pristane, boosted 2 days later with antigen and injected with 1×10^6 SP2/0 non-secreting myeloma cells after a further 7 days. Mice were subsequently monitored and ascitic fluid was harvested. The mouse used for the present experiments yielded 6 ml of serum which recognised a single 25 kDa band on immunoblots of nuclear proteins (data not shown, but supplied to referees); it was used for immunofluorescence at a dilution of 1/1000.

The secondary antibodies and the dilutions used were as follows: FITC-conjugated sheep anti-mouse IgG and goat anti-rabbit IgG (Sigma), both at 1/100; Cy3-conjugated goat anti-rabbit (1/2000), goat anti-rat (1/500) and goat anti-mouse (1/500); Cy5-conjugated goat anti-mouse and goat anti-rabbit (1/500). All Cye-dye reagents were obtained from Amersham.

Immunofluorescence

Cells on coverslips in Linbro wells (seeded at a density of 1×10^5 cells per well) were fixed with formaldehyde (5% v/v in phosphate-buffered saline (PBS) containing 2% sucrose), then permeabilised with 0.5% NP40 in PBS with 10% sucrose. After washing in PBS/1% calf serum, the coverslips were placed cell side down on 20 μl droplets of antibody dilutions in PBS/1% calf serum on a clean plastic surface. After incubation at room temperature for 1 hour, the coverslips were washed at least 6 times and incubated with secondary antibodies in a similar manner. After a further 60 minute incubation, the coverslips were again washed at least 6 times and mounted using Citifluor. When cells were triple labelled with mouse, rat and rabbit sera, the staining order was: (1) primary mouse and rabbit sera; (2) secondary conjugated anti-mouse and anti-rabbit antibodies; (3) primary rat serum; (4) anti-rat secondary conjugated antibody. Control experiments indicated that this staining protocol avoided cross reaction between the mouse and rat reagents.

Microscopy

The samples were examined using a Zeiss LSM 510 confocal microscope with three lasers giving excitation lines at 633 nm, 543 nm and 488 nm. The data from the channels was collected either simultaneously or, when necessary, separately, using the narrow band pass filter settings built into the instrument. Double labelling experiments often employed a combination of FITC and Cy5 dyes to eliminate channel overlap effects. When Cy3 and FITC were used for double staining, the laser intensities and data collection settings were adjusted so that channel overlap was undetectable, or the data were collected separately. Specimens were tested for overlap by turning off individual laser lines while scanning continuously. Data was collected with 8-fold averaging at a resolution of 1024×1024 pixels using optical slices of about 0.5 μm. The microscope was a Zeiss Axioplan

utilising 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.

RESULTS

hDaxx and HP1 are components of both ND10 and centromeres

Because HSV-1 regulatory protein Vmw110 associates with both ND10 and centromeres and induces the proteasome-dependent degradation one or more of their component proteins, it seemed possible that a hitherto unsuspected link exists between these apparently distinct nuclear substructures.

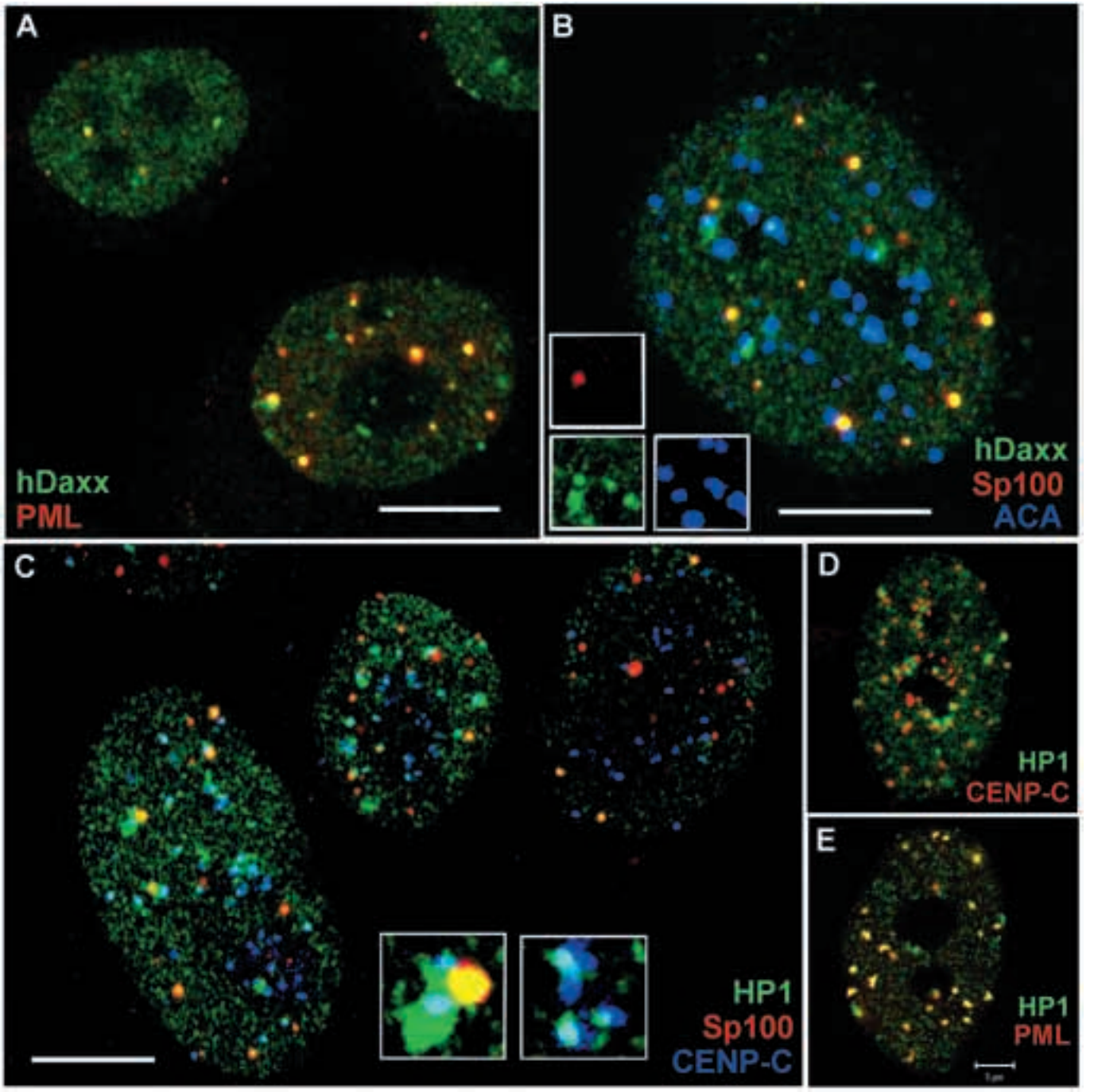
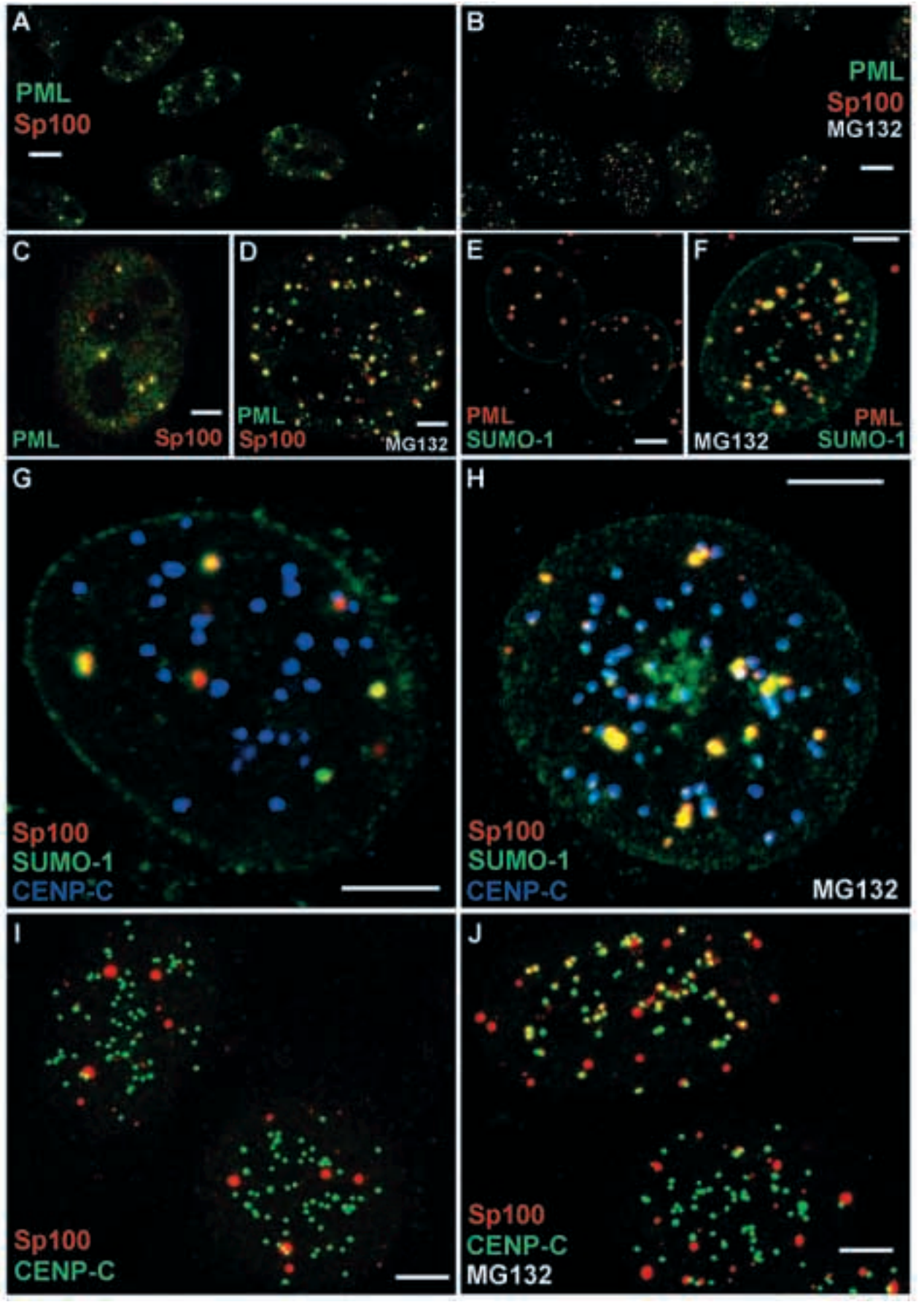


Fig. 1. hDaxx and HP1 can be associated with both ND10 and centromeres. Hep 2 cells were stained as indicated by the annotations. (A and B) Unsynchronised interphase cells; (C) S phase cells giving a spread of phenotypes; (D and E) S phase and G_2 cells with prominent association of HP1 with centromeres and ND10, respectively. The insets are explained in the text. Bars: 10 μm (A,B,C); 5 μm (E).


A
PML
Sp100
B
PML
Sp100
MG132
C
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D
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MG132
E
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SUMO-1
F
MG132
PML
SUMO-1
G
Sp100
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CENP-C
H
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SUMO-1
CENP-C
MG132
I
Sp100
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MG132

One approach to investigate this possibility was to determine whether there are proteins which are present in both structures. We chose to investigate two likely candidates: hDaxx, the human homologue of the murine apoptosis regulator Daxx, interacts with centromere protein CENP-C and immunolocalises at interphase centromeres and uncharacterised punctate nuclear foci (Pluta et al., 1998); heterochromatin-associated protein HP1 interacts with ND10 protein Sp100 (Seeler et al., 1998; Lehming et al., 1998) and associates with centromeres during mitosis (Wreggett et al., 1994; Furuta et al., 1997).

Hep2 cells stained for hDaxx and PML showed that hDaxx has a granular nuclear staining pattern with localised foci of increased intensity, the number of which varied from cell to cell. Some of the hDaxx foci co-localised with PML in ND10, but in many cells there were additional regions of hDaxx accumulation which were often more diffuse than the ND10 (Fig. 1A; see also Fig. 5J). Triple labelling with human anti-centromere, rabbit anti-hDaxx and mouse anti-PML antibodies showed that centromeres were often associated with hDaxx foci, but this again varied between cells. There were also additional hDaxx foci which were not apparently associated with either ND10 or centromeres (Fig. 1B; see Fig. 5G for an example of a cell double stained for hDaxx and centromeres). The insets in Fig. 1B show the single channel data from a region in the upper left part of the cell, with an ND10 domain containing hDaxx (yellow in the triple channel image), four centromeres with hDaxx (light blue) and three without (dark blue). These results confirm the previous findings on the association of hDaxx with centromeres in a proportion of interphase cells and define some of the additional hDaxx foci as ND10.

Similar results were obtained with a murine polyclonal serum which recognises the α isoform of human HP1. Staining of interphase cells with this antibody gave a bright, granular nuclear staining pattern with embedded regions of more intense staining, the number of which varied between cells. Synchronised cells in early G_1 displayed diffuse staining, then the localised concentrations become more pronounced as the cell cycle progressed (data not shown). Triple labelling experiments demonstrated that HP1 could be associated with both ND10 and centromeres (Fig. 1C); the left-hand inset in this panel shows a detail of the left-hand cell where an ND10 containing HP1 (yellow) is juxtaposed to a large accumulation of HP1 (green) within which are two centromeres (light blue). The right-hand inset shows a detail from the middle cell illustrating close association of HP1 with three centromeres. Double staining experiments of synchronised cells showed that the localised concentrations of HP1 were predominantly associated with centromeres in S phase (Fig. 1D), but many cells in G_2 displayed striking co-localisation of PML and HP1 (Fig. 1E). It had previously been shown that HP1 co-localised with ND10 after extraction of soluble protein before fixation (Seeler et al., 1998). Our results extend this finding to demonstrate that localised concentrations of HP1 associate with both centromeres and ND10 in interphase cells. Therefore there are at least two proteins, hDaxx and HP1, that accumulate at both ND10 and centromeres.

Fig. 2. Treatment with MG132 affects the relative distribution of PML, Sp100 and SUMO-1 and leads to association of ND10 proteins and SUMO-1 with centromeres. (A and B) Typical views of untreated cells (A) or cells treated with MG132 (5 μM) for 4 hours (B) stained for PML with r8 serum (green) and Sp100 with rat serum sp26 (red). (C and D) Control and MG132-treated cells at higher magnification; note the appearance of dots containing only PML or Sp100, and the partial separation of the PML and Sp100 signals (D). (E to H) SUMO13 cells stained as indicated; the characteristic presence of SUMO-1 at the nuclear membrane due to its conjugation to RanGAP1. The yellow dots in the cell pair in E have SUMO-1 present at ND10. After treatment with MG132 (F) the number of PML-containing foci and their content of SUMO-1 increases, and there are also many novel punctate foci of SUMO-1 which are not associated with PML. (G) An untreated SUMO13 cell triple labelled as indicated. Note the variable content of SUMO-1 in ND10 but no SUMO-1 in centromeres. (H) A typical SUMO13 cell after treatment with MG132 (5 μM for 2 hours). Consistent with panel F, there are many novel foci of SUMO-1, but these are present at both ND10 (yellow) and at centromeres (white/light blue modification of the blue staining). Note that some structures contain all three proteins, but others have variations of combinations of two of the three proteins. (I and J) Association of Sp100 with centromeres in MG132-treated cells. Cells stained for Sp100 with rat sp26 (red) and for CENP-C with rabbit r554 (green) sera either untreated (I) or after treatment with 5 μM MG132 for 4 hours (J). The images are z-series optical slices projected onto a single plane to include all the ND10 and centromere structures in the cell. Examination of single optical slices derived from image (I) indicated that some of the apparent Sp100-centromere associations in this projection were not found in the single optical slices (not shown). (J) An example of a MG132-treated cell with widespread Sp100-centromere association, which is a true representation of the association seen in the single optical slices used for this projection. The lower cell in J is an example of a cell in which the Sp100 and CENP-C signals have remained separate. Bars: 5 μm (A and B, 10 μm).

Increase in the number of foci of ND10 proteins induced by the proteasome inhibitor MG132

During previous studies on the effect of virus infection on ND10, we noted that treatment of cells with the proteasome inhibitors MG132 and lactacystin caused an apparent increase in the number of ND10 (Everett et al., 1998a). To investigate this effect in more detail, we examined Hep2 cells after treatment with MG132 for increasing times. The results confirmed that MG132 increased the apparent number of ND10 detected by both anti-PML and anti-Sp100 antibodies (Fig. 2A,B). The effect of MG132 on ND10 number was established after about 2 hours of treatment, becoming maximal by 4 hours and not increasing thereafter (data not shown). The caspase inhibitor zVAD caused no alterations to ND10, showing that the MG132 effect was not a non-specific consequence of inhibition of cellular proteases (Fig. 5C). Despite the changes in ND10 in response to MG132 observed by microscopy, western blotting showed that the drug had little effect on the amounts of the various modified isoforms of PML and Sp100, although in some experiments there was a slight increase in the higher molecular mass modified forms of both proteins (data not shown, but see Everett et al., 1998a for examples of data with PML). Therefore neither protein is rapidly turned over by the ubiquitin-proteasome pathway under normal conditions, and the increased number of ND10 foci represents redistribution of existing protein rather than an increase in the quantity of the ND10 proteins themselves. The increased numbers of PML and Sp100 foci may indicate sites where the

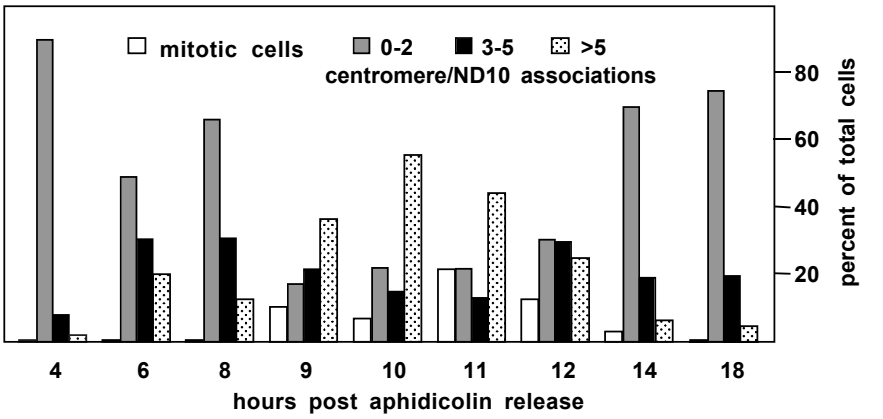


Fig. 3. Numerical analysis of ND10-centromere association in MG132-treated cells at different stages of the cell cycle. MG132 treatment of Hep2 cells was commenced at the indicated times after release from an aphidicolin block, the cells were fixed 2 hours later, stained for Sp100 and CENP-C, then two random fields of cells (using the ×40 objective lens) were scanned separately for the two channels. The total number of cells examined for each time point was between 50 and 80, and these were scored for having 0-2, 3-5 and greater than 5 instances of ND10-centromere association. The results were normalised as percentages of the total number of cells counted.

presence of ND10 components is normally transient as a result of proteasome-mediated turnover of another protein.

MG132 induces the partial separation of PML and sp100

PML and Sp100 are tightly associated in ND10 in interphase cells, and double staining shows predominantly precise co-localisation of the punctate signals of both proteins (Fig. 2A,C). However, after treatment with MG132, although the number of foci of both PML and Sp100 increased, we found that a number of Sp100 accumulations appeared which contained little or no PML (Fig. 2B,D). Corresponding foci of PML which did not contain Sp100 were also found. Furthermore, even the major sites containing both PML and Sp100 were noticeably different from ND10 in untreated cells since instead of precisely co-localising, the PML and Sp100 signals frequently only partially overlapped (Fig. 2D; compare with the control Fig. 2C). Identical results were obtained using different combinations of primary and secondary antibodies (data not shown). These findings emphasise the dynamic state of ND10, demonstrating that as well as being variable in number, the composition and structure of ND10 can also be changed so that even two of the most tightly linked constituent proteins can be separated. Therefore it is clear that there are other sites in the nucleus where PML and Sp100 can accumulate, and that these sites need not necessarily contain both of the major ND10 proteins.

Detection of SUMO-1 in both ND10 and centromeres

Because HSV-1 regulatory protein Vmw110 induces the degradation of CENP-C and the likely SUMO-1 conjugated forms of PML and Sp100, we have previously suggested that it is feasible that the ND10 proteins and CENP-C carry the same or a similar modification (Everett et al., 1999). Previous work has shown that SUMO-1 co-localises with PML in ND10 (Boddy et al., 1996; Sternsdorf et al., 1997a,b; Muller et al., 1998), but there has been no evidence that SUMO-1 could also be present in centromeres. To investigate this point in more detail we utilised a cell line expressing an epitope tagged version of SUMO-1, thereby taking advantage of the cleaner fluorescence and greater sensitivity of the anti-tag monoclonal compared to the available anti-SUMO-1 reagents. Initial experiments showed that the tagged SUMO-1 expressed by these cells was present at both the nuclear membrane and ND10, as expected (Fig. 2E), but there was no obvious staining at centromeres (Fig. 2G). However, following treatment with MG132, SUMO-1 was present in an increased number of intranuclear foci (Fig. 2F) and triple labelling demonstrated that these foci coincided with both centromeres and ND10 (Fig. 2H). The most likely explanation of this result is that MG132 stabilises a normally short-lived association of SUMO-1 with centromeres, and that both ND10 and centromeres have connections with SUMO-1 modification pathways.

Juxtaposition of centromeres and ND10 proteins in a proportion of MG132-treated cells

As explained above, these studies arose from our observations on the effect of HSV-1 infection on ND10 and centromeres, and the Vmw110-induced proteasome-dependent degradation of centromeric protein CENP-C and the ND10 components PML and Sp100 (Everett et al., 1998a, 1999). We had found that MG132 protected both of these structures from the effects of Vmw110 and caused extensive, stable association of Vmw110 with ND10 (Everett et al., 1998a). In separate double-labelling experiments we found that some MG132-treated cells had significant numbers of centromeres with associated Vmw110 (Everett et al., 1999). Because Vmw110 was present in ND10 in all MG132-treated cells, and also at centromeres in a subset of such cells, the implication was that centromeres and ND10 may be associated in this subset of MG132-treated infected cells. Therefore we investigated this potential connection between ND10 proteins and centromeres in uninfected cells by treating with MG132 to increase the number of foci of ND10 proteins, then screening fields of cells to search for any in which centromeres and ND10 proteins were associated.

In the absence of MG132, Hep2 cells contain a normal complement of centromeres and a much smaller number of ND10. As observed previously (Ascoli and Maul, 1991; Stuurman et al., 1992), examples of ND10 being juxtaposed to centromeres could occasionally be found (Fig. 2I), but this was uncommon and was likely to have occurred by chance. In the presence of MG132, however, although in most cells ND10 and centromeres remained largely separate, in some cells there was striking association between many punctate foci containing either Sp100 (Fig. 2J) or PML (Fig. 5A) and centromeres. Since examples of these cells could be found using different combinations of antibodies it is highly unlikely that this co-localisation occurred by chance. The most likely explanation is that there is normally a transient, dynamic connection

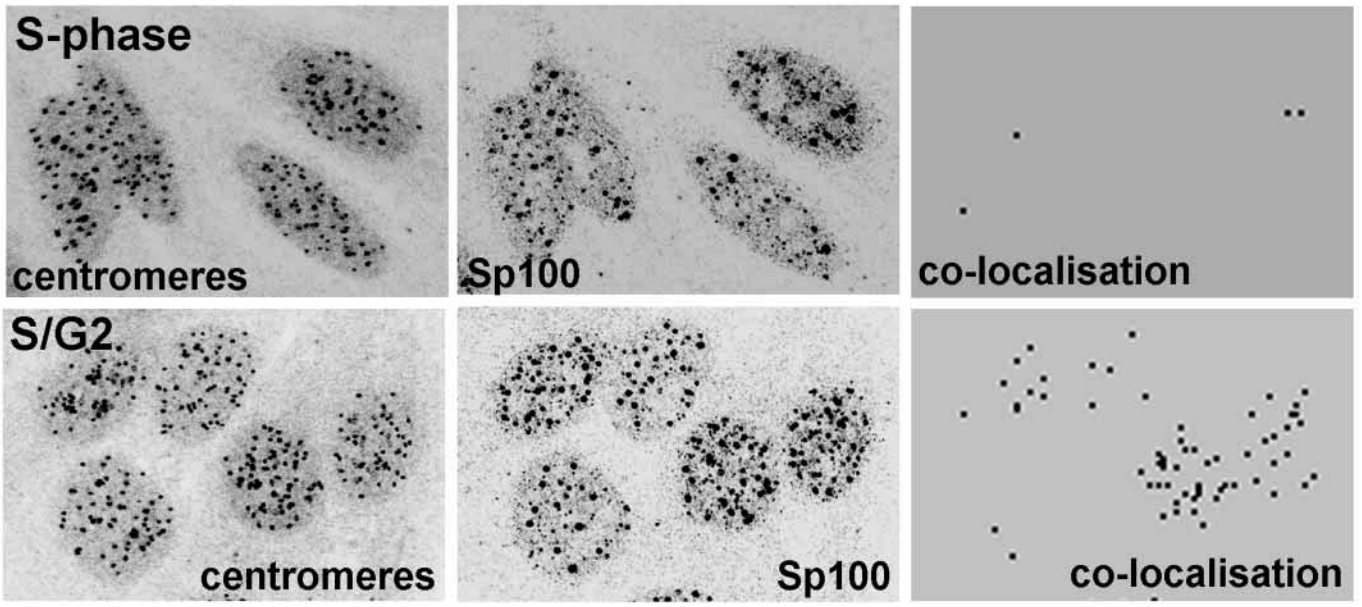


Fig. 4. Detailed analysis of Sp100-centromere association in MG132 treated synchronised cells. Cells were treated with MG132 for 2 hours at 4 hours (top row; S phase) and 10 hours (S/G_2; bottom row) post aphidicolin release, then stained for Sp100 and CENP-C. Single confocal slice images (about 0.8 μm) were obtained from random fields of cells, then the red and green channels were converted individually to greyscale and the number of dots counted. Co-localisation was determined using LSM 510 software, which identifies all pixels in which both red and green signals are within 30% of the maximum for that channel and outputs this information in a superimposed channel. The co-localisation image data was converted to greyscale. The figure shows representative segments of the images from the 4 hours and 10 hour time points at the magnification used for numerical analysis.

between ND10 proteins and centromeres which can be stabilised in the presence of MG132. Although cells with extensive association between ND10 and centromeres were rare (approximately 2 to 5% of the total), the effect of MG132 was reproducible over a large number of experiments and in different cell types.

The low frequency of ND10/centromere association, even in the presence of MG132, suggested to us that this might occur preferentially at certain times in the cell cycle. To test this hypothesis, we repeated the experiment by treating synchronised cells with MG132 at intervals after release from an aphidicolin block. Cells were double stained for Sp100 and CENP-C, then images of two random fields of cells were captured for further study. The images were initially scored by visual examination for the percentage of mitotic cells to control for synchronisation, and for the number of centromere/Sp100 associations in individual cells. The results showed that cells with increased MG132-induced association between Sp100 and centromeres became more abundant as the cell cycle progressed (Fig. 3). The MG132-induced increase in the number of Sp100 foci was itself cell cycle regulated, being much less pronounced in early G_1 compared to S phase and G_2 (data not shown). Because cells in G_2 had much greater numbers of Sp100 foci, it was possible that the numbers of Sp100/centromere associations had been increased by chance. Therefore images of cells in S phase and G_2 were examined in more detail. Total numbers of centromeres and Sp100 foci in the single confocal optical slices were counted after conversion of the separate channels to greyscale images, then the number of co-localising foci was determined using software which identifies pixels with high intensity signals in both channels. Examples of segments of these data are shown in Fig. 4, and detailed analysis of two of the images are summarised in Table 1. The results clearly show that MG132-induced Sp100/centromere association was cell cycle dependent, was not proportional to the number of Sp100 foci, and that in some cells more than 40% of Sp100 foci were associated with centromeres.

Given the above results, we re-examined untreated synchronised cells for association of ND10 proteins with centromeres. We found that cells in late G_2 often had substantially increased numbers of ND10, and that rare cells could be found in which there was considerable association between ND10 and centromeres. Fig. 5B shows an example in which there are more than 20 instances of ND10-centromere association in an untreated G_2 cell. Although cells with this phenotype were in low abundance, this is an important result since it shows that the ND10-centromere association is not an artefact of exposure to MG132 and that inhibitor treatment probably reveals an interaction which is normally very short lived.

The results in Figs 3 and 4 could be explained if MG132 blocked cell cycle progression in G_2 at a stage when the probability of ND10-centromere association was high. However, we found that although MG132 slowed the progression of cells through S phase and G_2 into mitosis, it did not block the cell cycle at a defined point in G_2 (data not shown).

Increased foci of ND10 proteins induced by heat shock and interferon, and in promyleocytic leukaemia cells, do not result in association of ND10 with centromeres

Heat shock, interferon and expression of the PML-RARα

Table 1. Detailed analysis of Sp100 co-localisation with centromeres after treatment of synchronised cells with MG132

A: 4 hours post release					B: 10 hours post release				
Cell	CENP-C	Sp100	co-loc	percentage	Cell	CENP-C	Sp100	co-loc	percentage
1	46	39	8	21	1	47	44	25	57
2	38	16	3	19	2	56	24	10	42
3	47	54	7	13	3	33	31	13	42
4	52	43	2	5	4	48	27	11	41
5	48	27	1	4	5	48	48	19	40
6	43	34	1	3	6	46	50	20	40
7	45	30	1	3	7	45	50	17	34
8	44	30	1	3	8	18	19	6	32
9	48	31	1	3	9	37	36	10	28
10	48	33	0	0	10	35	35	9	26
11	24	10	0	0	11	51	44	11	25
12	48	16	0	0	12	35	43	10	23
13	50	19	0	0	13	44	44	9	20
14	43	38	0	0	14	34	36	7	19
15	38	5	0	0	15	27	34	6	18
16	40	14	0	0	16	30	34	6	18
17	63	46	0	0	17	22	18	3	17
18	32	31	0	0	18	25	18	3	17
19	53	39	0	0	19	47	44	7	16
20	43	33	0	0	20	34	37	6	16
21	35	33	0	0	21	40	28	4	14
22	46	32	0	0	22	20	24	3	13
23	33	19	0	0	23	38	30	4	13
24	38	18	0	0	24	24	23	3	13
25	37	19	0	0	25	24	28	3	11
26	41	13	0	0	26	27	17	1	6
27	34	24	0	0	27	37	40	1	3
Av.	**41.6**	**27.7**	**0.9**	**3**	**Av.**	**36.0**	**33.6**	**8.4**	**24**
s.d.	**8.8**	**11.4**	**1.9**	**5**	**s.d.**	**10.4**	**10.2**	**6.1**	**13**

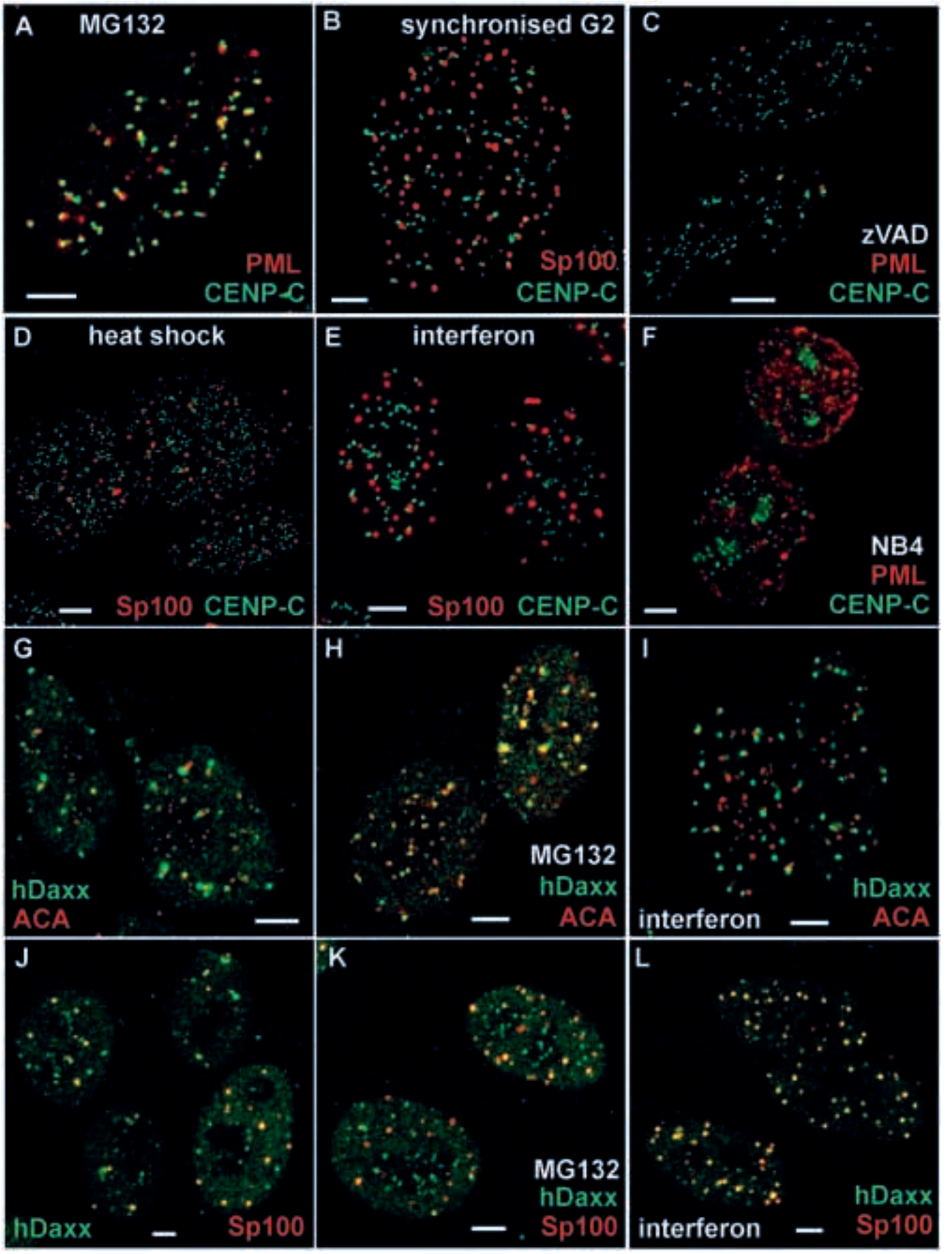
Notes: Cells treated with MG132 for 2 hours at 4 hours and 10 hours post aphidicolin release, then stained for Sp100 and CENP-C. Single confocal slice images (about 0.8 μm) were obtained from random fields of cells, then the red and green channels were converted to greyscale and the number of dots counted. Co-localisation (co-loc) was determined using LSM 510 software, as described in Fig. 4. The data presented are from single fields of cells, but essentially identical results were obtained using a second set of images. The percent column gives the percentage of Sp100 foci that are associated with centromeres.

fusion protein in promyelocytic leukaemia cells all increase the numbers of foci of ND10 proteins (reviewed by Sternsdorf et al., 1997a), but none of these treatments increased the association of Sp100 or PML with centromeres (Fig. 5D,E,F). These results support the conclusion that the association observed above is a specific consequence of MG132 treatment, rather than being caused by increased numbers of Sp100 foci.

Evidence for transfer of hDaxx between ND10 and centromeres

After examination of large numbers of cells, we considered it was possible that hDaxx has a dynamic relationship with ND10 and centromeres. This hypothesis was supported by examination of hDaxx distribution after treatment with interferon or MG132. Cells treated with interferon exhibited decreased hDaxx association with centromeres (Fig. 5I; note the decreased proportion of yellow dots compared to Fig. 5G), whereas hDaxx was associated with virtually all interferon-induced ND10 (Fig. 5L). In contrast, MG132 treated cells had increased hDaxx association with centromeres (Fig. 5H; note the high proportion of yellow centromere dots), but in most cells many of the foci of Sp100 induced by MG132 did not contain hDaxx (Fig. 5K). Triple labelling of MG132-treated cells showed that, consistent with the effect of MG132 described above, a small proportion of cells exhibited triple co-localisation of hDaxx, Sp100 and anti-centromere antibodies, thus confirming the previous result using a different

Fig. 5. Association of PML, Sp100 and hDaxx with centromeres after various treatments. Antibodies were used as described in materials and methods and in the text and were stained as indicated on the labels. The treatments are shown in white lettering: MG132, 5 μM; 2 hours, zVAD; 100 μM, 6 hours; heat shock: 42°C, 15 minutes; interferon, α-interferon (1000 units/ml) for 16 hours. (A,B,D,E) A series of confocal slices amounting to a depth of 1.5 μm projected onto a single plane to show most ND10 and centromere structures in this cell. (C and F) Produced using the Deltavision system, and represent deconvolved data from the whole depth of the cell projected onto a single plane. (B) A cell with more than 20 instances of Sp100-centromere association, each of which could be found in the single optical sections. (F) The distribution of PML and CENP-C in NB4 cells derived from a patient with promyelocytic leukaemia. Further details of the images are given in the text. The images show a typical results which were selected after scrutiny of large numbers of cells. Bars, 5 μm.


A MG132
PML
CENP-C
B synchronised G2
Sp100
CENP-C
C
zVAD
PML
CENP-C
D heat shock
Sp100 CENP-C
E interferon
Sp100 CENP-C
F
NB4
PML
CENP-C
G
hDaxx
ACA
H
MG132
hDaxx
ACA
I
hDaxx
interferon
ACA
J
hDaxx
Sp100
K
MG132
hDaxx
Sp100
L
hDaxx
interferon
Sp100

combination of antibodies (data not shown). These results show that the relative association of hDaxx with centromeres and ND10 can vary, thereby demonstrating a reciprocal link between the protein compositions of the two structures.

DISCUSSION

This paper demonstrates that hDaxx and HP1 are components of both ND10 and centromeres, and that an association between centromeres and the ND10 proteins PML and Sp100 occurs in a cell cycle dependent manner and is greatly augmented after inhibition of proteasome-mediated proteolysis. An emerging picture is that the composition of individual ND10 may be influenced by a variety of factors, including the normal progression of the cell cycle and external factors such as virus infection, heat shock and treatment with interferon and the proteasome inhibitor MG132. For example, both HAUSP and RFP are present in only a proportion of ND10 in a subset of unsynchronised interphase cells (Everett et al., 1997; Cao et al., 1998), and at least in the case of HAUSP this variation is controlled by the cell cycle (unpublished results). Furthermore, although Sp100 and PML are normally tightly associated in ND10 in interphase, they become separated during mitosis (Sternsdorf et al., 1997b). In a similar way, we found that the appearance of local accumulations of HP1 and their association with ND10 is cell cycle related. Thus several components of ND10 behave in a dynamic manner, being present not only in localised concentrations within ND10 but also at other locations in the nucleus. This study extends this principle to show that certain ND10 associated proteins can also be present at centromeres, or in their close vicinity.

A number of earlier studies concluded that ND10 were not obviously associated with sites of abundant transcription, DNA replication, splicing speckles, coiled bodies or centromeres (Ascoli and Maul, 1991; Stuurman et al., 1992; Weis et al., 1994). However, given the dynamic nature of the nucleus, it is possible that there may be biologically relevant functional connections between seemingly separate structures which can only be detected during a limited temporal window. As in previous studies which investigated the relative distribution of ND10 and centromeres, we found that in normal interphase cells these structures showed little evidence of any association. Although occasional juxtaposition of the two could be found, this involved only one or two domains in occasional nuclei, and did not appear to occur more frequently than would be predicted by chance. However, the present study has shown that proteasome inhibition with MG132 can result in significant association of Sp100 and PML with centromeres in many cells, particularly during the G_2 phase of the cell cycle. Follow-up studies of synchronised cells without any drug treatment confirmed that ND10 numbers are increased in some G_2 phase cells, and that in some of these cells large numbers of intimate ND10/centromere associations occur. This suggests that a short-lived association between some ND10 proteins and centromeres occurs during G_2. It is important to note that the association between ND10 proteins and centromeres does not arise simply by chance when MG132 increases the number of ND10 protein foci. In cells from an individual with acute promyelocytic leukaemia, the number of ND10 speckles is greatly increased, but no co-localisation is seen with centromeres (Fig. 5F).

Components of both centromeres and ND10 are targets for proteasome-dependent degradation induced by Vmw110, and because the ND10 components that are preferentially targeted are conjugated to SUMO-1, we have speculated that CENP-C may itself be modified by SUMO-1 or a similar protein (Everett et al., 1998a, 1999). If so, ND10 and centromeres could be connected through a common involvement in SUMO-1 modification pathways. This possibility is supported by the presence of SUMO-1 in centromeres in MG132-treated cells. It is therefore possible that the association of ND10 with centromeres caused by the drug arises through direct or indirect interactions between SUMO-1-modified components in these two compartments. Our data suggest that SUMO-1 modifications at centromeres are normally short-lived, and this could explain the very occasional cells in G_2 phase in which an association of the two structures can be seen in the absence of MG132.

The presence of HP1 and hDaxx in a proportion of both centromeres and ND10 in subset of cells provides further links between the two structures. Little is known of the functions of hDaxx. Murine Daxx was cloned after screening for proteins interacting with the death domain of Fas, and was shown to enhance Fas-mediated apoptosis (Yang et al., 1997). Subsequently hDaxx was cloned as a binding partner for centromere protein CENP-C (Pluta et al., 1998), and also as a DNA binding protein (Kiriakidou et al., 1997). The nuclear staining pattern of hDaxx has some similarities with that of HP1 (see below), which could indicate that it associates not only with ND10 and centromeres, but also the chromatin surrounding the centromeres. How its interaction with ND10 and other nuclear components relates to its reported role in Fas-mediated apoptosis, and whether there is any connection with the roles of PML in apoptosis (Wang et al., 1998b), remains to be determined. However, it is possible that targeting of CENP-C and PML by Vmw110 during HSV-1 infection somehow inactivates an apoptotic response triggered by hDaxx, thereby permitting the virus to complete a productive infection.

Another possible functional explanation of the association between ND10 proteins and centromeres is suggested by the properties of HP1, a protein that associates with centromeric heterochromatin and enhances the silencing phenomenon known as position effect variegation (PEV) in *Drosophila* (James et al., 1989; Eissenberg et al., 1990, 1992). It is intriguing that centromeres are frequently found in a larger region of HP1 staining (Fig. 1C). This could indicate that HP1 is also associated with the surrounding chromatin, a situation similar to that described for mammalian homologues of the PEV suppresser *Su(var)3-9* (Aagaard et al., 1999). It has been suggested that centromeric heterochromatin is able to recruit repressed genes into a transcriptionally inactive subnuclear compartment (Csink and Henikoff, 1996; Dernberg et al., 1996; Brown et al., 1997) and it is conceivable that the local accumulations of HP1 observed in the present study represent visualisation of such repression regions. Therefore the presence of HP1 in ND10 may indicate a connection between ND10 and gene silencing pathways, a possibility that is supported by recent evidence linking Sp100 with transcriptional repression (Lehming et al., 1998). This could explain why Vmw110 targets both ND10 and centromeres, it

could be inactivating a cellular repression mechanism to prevent the silencing of the viral genome that can occur at the early stages of HSV-1 infection (Everett et al., 1998b and references therein). This model suggests that future studies on the relative localisation of ND10, centromeres and other proteins implicated in repression pathways may be fruitful.

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