Mitotic phosphorylation of SUV39H1, a novel component of active centromeres, coincides with transient accumulation at mammalian centromeres

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SUMMARY

Centromeres of eukaryotes are frequently associated with constitutive heterochromatin and their activity appears to be coregulated by epigenetic modification of higher order chromatin. Recently, we isolated murine (Su39h1) and human (SUV39H1) homologues of the dominant Drosophila suppressor of position effect variegation Su(var)3-9, which is also related to the S. pombe silencing factor Cln4. We have shown that mammalian Su(var)3-9 homologues encode novel centromeric proteins on metaphase-arrested chromosomes. Here, we describe a detailed analysis of the chromatin distribution of human SUV39H1 during the cell cycle. Although there is significant heterochromatic overlap between SUV39H1 and M31 (HP1β) during interphase, mitotic SUV39H1 displays a more restricted spatial and temporal association pattern with metaphase chromosomes than M31 (HP1β), or the related HP1α gene product. SUV39H1 specifically accumulates at the centromere during prometaphase but dissociates from centromeric positions at the meta- to anaphase transition. In addition, SUV39H1 selectively associates with the active centromere of a dicentric chromosome and also with a neocentromere. Interestingly, SUV39H1 is shown to be a phosphoprotein with modifications at serine and, to a lesser degree, also at threonine residues. Whereas SUV39H1 steady-state protein levels appear constant during the cell cycle, two additional phosphorylated isoforms are detected in mitotic extracts. This intriguing localisation and modification pattern would be consistent with a regulatory role(s) for SUV39H1 in participating in higher order chromatin organisation at mammalian centromeres.

Key words: SUV39H1, Phosphorylation, Cell cycle, Centromere, Higher order chromatin

INTRODUCTION

The chromatin of multicellular eukaryotes is divided into euchromatic and heterochromatic domains. Whereas euchromatin generally is permissive for transcriptional activity, heterochromatin appears highly condensed, gene-poor and late-replicating, with described functions in epigenetic gene silencing and in the structural organisation of centromeres and telomeres (Karpen and Allshire, 1997; Grunstein, 1998). In particular, centromeres are usually surrounded by large blocks of constitutive heterochromatin, which has been suggested to nucleate centromere activity (Csink and Henikoff, 1998). Indeed, epigenetic marking rather than a specific DNA sequence appears to underlie a functional centromere (Ekwall et al., 1997), since some human dicentric chromosomes can be stably segregated, and sequences with no detectable α-satellite DNA can occasionally adopt neocentromere activity (du Sart et al., 1997; Williams et al., 1998).

A variety of mammalian centromeric proteins have been identified (Choo, 1997). Among these, the constitutive proteins CENP-A, B, C and G appear to be structural centromeric components, since they are associated with the centromere throughout the cell cycle (Dobie et al., 1999). CENP-A represents a histone H3 variant that may induce an altered nucleosomal array at the centromere (Shelby et al., 1997). CENP-B is a DNA binding protein that recognises target sites in α-satellite sequences, but whose presence is not strictly required for centromere function (Hudson et al., 1998). The exact role of CENP-C is unclear, but gene disruption of CENP-C leads to severe mitotic defects (Kalitsis et al., 1998). A number of motor proteins are also cytologically visible at the outer centromere region during mitosis, one of the best characterised being the plus-end directed, kinesin-like motor protein CENP-E (Wood et al., 1997). Additionally, components of the mitotic checkpoint machinery, the MAD/BUB pathway, which monitors correct chromosome alignment before allowing entry into anaphase, are specifically associated with the centromeres of unattached chromosomes (Li and Benezra, 1996; Taylor and McKeon, 1997).

A different class of centromere-associated proteins with
Fig. 1. Chromatin distribution of SUV39H1 during the cell cycle. Exponentially growing HeLa cells were hypoton-treated, spread by cytocentrifugation and probed with α-Suv39h1 antibodies by triple-labelling (yellow). DNA was counterstained by DA-DAPI (blue). The SUV39H1 signals are artificially coloured yellow to enhance visibility of weakly stained areas.
Mitotic phosphorylation of SUV39H1 currently less well defined functions are encoded by some suppressors of position effect variegation [Su(var)]. Several loci that influence centromeric silencing have been identified by genetic screens both in S. pombe (Allshire et al., 1995) and in Drosophila (Reuter and Spierer, 1992; Wallrath, 1998). Mutations in the S. pombe genes Swi6 and Clr4 alleviate

Fig. 2. Colocalisation of SUV39H1 with M31 (HP1β) and HP1α. (A) Exponentially growing HeLa cells were hypoton-treated, spread by cytocentrifugation and sequentially incubated with α-M31 (red) and α-Suv39h1 (yellow) antibodies. DNA was counterstained with DA-DAPI (blue). (B) Colcemid-arrested HeLa cells were hypoton-treated, spread by cytocentrifugation and sequentially incubated with α-HP1α (red) and α-Suv39h1 (yellow) antibodies. The SUV39H1 stains are artificially coloured yellow. Inserts show representative distribution of antigens at enlarged chromosomes.
centromeric gene silencing and lead to chromosome segregation defects, implying involvement in forming a fully functional centromere (Allshire et al., 1995; Ekwall et al., 1996). Recently, it has been shown that Swi6 is synthetic lethal with Bub1, indicating that Swi6-dependent defects can be suppressed by the MAD/BUB pathway (Bernard et al., 1998). The Swi6 homologue in Drosophila is the Su(var)2-5 gene, encoding heterochromatin protein 1 (HP1) (Eisenberg et al., 1992). Mutational analyses indicate a major function for HP1 in coregulating higher order chromatin at centromeres and telomeres (Fanti et al., 1998).

In contrast to these experimental model systems, SU(VAR)616 protein function in mammals is largely unknown. Thus far, three mammalian HP1-like proteins, designated HP1α, HP1β (M31) and HP1γ (M32), have been described (Saunders et al., 1993; Wreggett et al., 1994; Horsley et al., 1996). With the exception of M32, mammalian HP1 proteins preferably localise to pericentric heterochromatin, although they have several weaker binding sites along the arms and at the telomeres (Wreggett et al., 1994; Furuta et al., 1997; Mine et al., 1997). Recently, we reported the isolation of murine (Suv39h1) and human (SUV39H1) homologues (Aagaard et al., 1999) of Drosophila Su(var)3-9 (Tschiirsch et al., 1994), which is also related to the S. pombe silencing factor Ctr4 (Ivanova et al., 1998). Members of the SU(VAR) protein family are of particular interest, because they combine the two most characteristic domains of chromatin regulators: the chromo and the SET domains (Jenuwein et al., 1998). The chromo domain appears to be a protein interaction motif (Ball et al., 1997) that directs heterochromatins and euchromatin associations (Messmer et al., 1992; Platero et al., 1995). The function of the SET domain is currently undefined, although it has been implicated as a target in phosphorylation-dependent signalling pathways that trigger proliferation or differentiation (Cui et al., 1998). M31 (HP1β) and SUV39H1 partly coimmunoprecipitate and cosediment, indicating the existence of a mammalian SU(VAR) protein complex (Aagaard et al., 1999). Su(var)3-9/SUV39H1 encode heterochromatic proteins, which are centromeric on metaphase-arrested human chromosomes (Aagaard et al., 1999). Here, we describe a detailed analysis of the chromatin distribution of SUV39H1 during the cell cycle. SUV39H1 is centromeric only during prometa- to metaphase, revealing a novel temporal distribution profile at mammalian centromeres. In addition, SUV39H1 selectively associates with the active centromere of a dicentric chromosome, and also with a neocentromere. Intriguingly, we demonstrate that the dynamic cell cycle distribution correlates with mitosis-specific phosphorylation of SUV39H1 of at least two sites. Together, our data support a model in which SUV39H1 may even play a regulatory role(s) in centromere-dependent mitotic chromosome organisation.

**MATERIALS AND METHODS**

**Antibodies**

Rabbit polyclonal α-Suv39h1 antibodies (Aagaard et al., 1999) were used at 1:10 dilution for immunofluorescence and 1:500 for western blotting. Rat monoclonal α-M31 antibodies (kindly provided by Prim Singh) were used as a crude tissue culture supernatant diluted 1:40 for western blot, and as a protein-A purified IgG fraction diluted 1:5 for immunofluorescence. Mouse monoclonal α-HP1α antibodies (kindly provided by Pierre Chambon) were diluted 1:2,000, human hACA antibodies (SM serum; kindly provided by K. Sullivan) 1:800, and mouse monoclonal α-dynein antibodies (kindly provided by Walter Steffen) 1:100, for immunofluorescence analysis. Mouse monoclonal α-tubulin antibodies (Sigma) were used at 1:10,000 dilution for western blot analysis. All secondary antibodies were purchased from Jackson Immuno Research Laboratories except when mentioned otherwise.

**Tissue culture, cell cycle synchronisation and FACS analysis**

HeLa and Brd cells were grown in Dulbecco’s modified Eagle medium (DMEM), 8828 cells in RPMI, both supplemented with 10% fetal calf serum at 37°C with 5% CO2. To synchronise HeLa cells in S phase, cells were cultivated for 18 hours in the presence of 2 mM thymidine, released for 8 hours, arrested with 2.4 mM thymidine for 18 hours and then shifted into fresh DMEM (time point 0). To synchronise HeLa cells in metaphase, cells were cultivated in the presence of nocodazole (0.1 μg/ml) for 18 hours and then harvested by mitotic shake-off. For FACS analysis, cells were fixed in cold 70% methanol, resuspended in 10 mM Tris pH 7.5, 5 mM MgCl2, 50 μg/ml propridium iodide, 10 μg/ml RNaseA, and incubated for 15 minutes at 37°C. Subsequently, DNA content of individual cells was determined using a FACScan (Becton Dickinson).

**Immunocytochemistry**

Immunofluorescence of unfixed human chromosomal spreads was essentially as described previously (Aagaard et al., 1999), with the following exceptions. Exponentially growing HeLa cells were harvested by mitotic shake-off before hypotonic treatment. For detection of low-abundant SUV39H1, samples were triple-labelled by sequential incubation with α-Suv39h1, CY-3 conjugated goat-α-rabbit and CY3-conjugated donkey-α-goat antibodies (Fig. 1). For colocalisation studies, slides were first incubated with either α-M31, α-HP1α, hACA or α-dynein antibodies, followed by CY3-conjugated goat-α-rat and CY3-conjugated donkey-α-goat antibodies (for M31 epitopes), by CY3-conjugated goat-α-mouse antibodies (for HP1α epitopes), by FITC-conjugated donkey-α-human antibodies (for CENPs epitopes) or by FITC-conjugated sheep-α-mouse antibodies (for dynein epitopes). Subsequently, the slides were incubated with α-Suv39h1 antibodies, followed by either Alexa 488-conjugated goat-α-rabbit (Molecular Probes) or CY3-conjugated goat-α-rabbit antibodies. To compensate for the reduced SUV39H1 signals as compared to the triple staining, longer exposures were processed for imaging analysis. Colcemid-arrest to enrich for metaphase cells was done as described (Aagaard et al., 1999).

**Immuo-FISH of human dicentric and neocentric chromosomes**

The human dicentric cell line 8828 carries a Robertsonian translocation of chromosomes 14 and 15, with the active centromere being derived from chromosome 14 (Sullivan and Schwartz, 1995). Human neocentric Brad cells carry an inversion-duplication (pter-13q21:1321-13qter), with the neocentromere being derived from chromosome 13 sequences (Warburton et al., 1997). Slides stained with α-Suv39h1 were RNaseA treated (0.1 μg/ml), refixed in 4% formaldehyde in 2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7), dehydrated in 70%, 80% and 100% ethanol, denatured in 70% formamide/2× SSC at 70°C and again dehydrated in 70%, 80% and 100% ice-cold ethanol. DIG-labelled probes recognising either chromosome 14/22 α-satellite (Oncor) or chromosome 15 α-satellite (Oncor) were diluted in 2× SSC, 50% formamide, 10% dextran sulphate, 1 μg/ml salmon sperm DNA and denatured at 95°C for 5 minutes. DIG-labelled COATASOME chromosome 13 probe (Oncor) was denatured at 70°C for 10 minutes and subsequently incubated at 37°C for 30 minutes. Slides with probes were again denatured for 10 minutes at 80°C before incubation at 37°C overnight. Slides were then washed in 65% formamide/2× SSC at 42°C and in 2× SSC at 37°C.
Probes were detected with FITC-conjugated sheep α-DIG antibodies (Boehringer Mannheim).

**2-D gel electrophoresis and phosphatase treatment**

Whole-cell protein extracts were prepared by boiling cells for 10 minutes in 1% SDS, 1 mM DTT, followed by 1 minute's centrifugation at room temperature. Protein concentration in the supernatant was determined using the D$_2$ protein assay (Biorad). Prior to phosphatase treatment, the lysates were dialysed overnight against phosphatase buffer (50 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 5 mM DTT), then supplemented with 0.01% Brij 35, 2 mM MnCl$_2$ and lysates treated with 4,000 U λ-phosphatase (New England Biolabs) for 1 hour at 30°C.

For 2-D analysis, 500 µg total protein was precipitated with methanol/chloroform (Wessel and Fluegge, 1984). Pellets were resuspended in 50 µl 9M urea, 4% Chaps, 65 mM Tris, 0.025% Bromophenol Blue (BBP), then 80 µl 8 M urea, 4% Chaps, 50 mM DTT, 2 M thiourea, 40% Resolyte pH 4-8 (Electran), 1% Resolyte 3.5-10 (Electran), 0.025% BBP was added. Proteins were focused on a non-linear Immobiline strip pH 3-10 (Pharmacia) with a total of 15,000 Vhours in an IPGphor chamber (Pharmacia). After equilibration in 6 M urea, 2% SDS, 30% glycerol, 100 mM Tris-HCl, pH 6.8, supplemented first with 2% DTT, then with 2.5% iodoacetamide, the strip was transferred to a polyacrylamide gradient gel (9-16%), and the proteins were separated according to size in the second dimension. Proteins were transferred to a PVDF membrane (Millipore) by semidry transfer in 10 mM CAPS, pH 11, 10% methanol.

For 1-D analysis, 80 µg total protein was mixed with 0.25 vol. 5% SDS, 40% glycerol, 397 mM DTT, 60 mM Tris, pH 6.8, 0.004% BBP, separated by SDS-PAGE and blotted to a PVDF membrane by semidry transfer in 48 mM Tris, 39 mM glycerol, 0.037% SDS, 20% methanol. After transfer, the membranes were probed with either α-Suv39h1, β-tubulin or α-M31 antibodies, followed either by horseradish peroxidase (HRP)-α-rabbit, HRP-α-mouse or HRP-α-rat secondary antibodies. Blots were developed using ECL (Amer sham).

**Flag-tagged SUV39H1 constructs**

A flag-tagged variant full-length (comprising amino acids 3-412) SUV39H1 cDNA was constructed by replacing the (myc)$_3$H$_6$ tag in CMV-(myc)$_3$H$_6$-SUV39H1 (Aagaard et al., 1999) with an oligonucleotide encoding a single flag epitope (DYKDDDDK) that specifically recognised a unique in-frame NotI site. To generate flag-tagged constructs comprising the chromo or the SET domain, PCR amplicons encoding amino acids 3-118 (Nchromo) or amino acids 161-412 (extended SET) were inserted into amplicons encoding amino acids 3-118 (Nchromo) or amino acids 161-412 (extended SET) were inserted into NotI-EcoRI or NotI-SphI digested CMV-flag-SUV39H1 vector DNA. Since the extended SET domain lacks the putative nuclear localisation signal (nls) present at amino acid positions 105-109 in SUV39H1 (Aagaard et al., 1999), a NotI oligonucleotide encoding the SV40 Nls (PKKKRKV) was additionally inserted in-frame into CMV-flag-SET to generate CMV-flagSET. All flag-tagged SUV39H1 inserts were confirmed by sequencing.

**In vivo labelling and immunoprecipitation of flag-tagged proteins**

2x10$^7$ HeLa cells were transiently transfected by electroporation with 20 µg of flag-tagged SUV39H1 constructs using a pulse of 270V/250 µF, cultured overnight and split into a T75 flask to reach ≈75% confluency. 36 hours after transfection, cells were washed in phosphate-free DMEM medium and labelled in vivo for 6 hours by incubation in 2 ml phosphate-free DMEM medium containing 10% FCS (which had been dialysed against TBS to remove phosphates) and 4 µCi ortho-phosphate ($^{32}$P; New England Nuclear). Cells were washed in TBS, harvested into 400 µl SDS lysis buffer (0.5% SDS, 0.05 M Tris-HCl, pH 8.0, 1mM DTT) and lysed by boiling for 5 minutes. The lysate was diluted to 2 ml RIPA buffer [1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA plus a full set of protease inhibitors (Boehringer Mannheim)], cleared by centrifugation and pre-incubated for 30 minutes at 4°C with 20 µl protein G-sepharose (Pharmacia). Flag-tagged proteins were immunoprecipitated with 20 µl α-flag M2 antibody beads (Sigma) for 2 hours at 4°C, washed six times in RIPA buffer, eluted by boiling for 5 minutes in SDS-sample buffer, separated by SDS-PAGE, transferred to a PVDF membrane (Millipore) and processed for immunoblotting with α-flag M2 antibodies (Sigma) or for autoradiography using an intensifying screen.

**Phospho-amino acid analysis**

Phospho-labelled flag-tagged SUV39H1 proteins were excised from the PVDF membrane, soaked in methanol, washed and hydrolysed in 6 M HCl for 1 hour at 110°C (following instructions described by Sefton, 1997). Solutions were dried in a speedvac (Savant), resolved in 10 µl water, separated by 2-D thin layer electrophoresis and processed for autoradiography. The identity of phosphorylated amino acids was confirmed by aligning the autoradiogram with ninhydrin-stained positions of a standard mixture of phosphate-serine, phosphothreonine and phospho-tyrosine (Sigma). The autoradiograms were exposed for 3 hours to several days using an intensifying screen.

**RESULTS**

**Dynamic chromatin association of SUV39H1 during the cell cycle**

We have shown previously that SUV39H1 localises to the centromeres in HeLa cells, if arrested in metaphase by microtubule destabilising drugs (colcemid) (Aagaard et al., 1999). To exclude dependence on drug treatment, we immunolocalised SUV39H1 on chromosomal spreads from untreated, exponentially growing HeLa cells. Again, SUV39H1 associates with the centromeres (Figs 1-3), indicating that the centromere-specific accumulation is independent upon the absence or presence of microtubules. In HeLa interphase cells, a very weak nuclear stain is detected with α-Suv39h1 antibodies, which is not specifically localised to constitutive heterochromatin, as visualised by the bright distamycin A-DAPI (DA-DAPI) counterstaining. In contrast to murine cells, the cytologically visible pericentric heterochromatin in human cells is very limited, and other heterochromatic proteins, like M31, are also not restricted to the DA-DAPI bright foci in HeLa cells (data not shown) (Minc et al., 1999). At the beginning of DNA condensation (prophase), the SUV39H1 stain is still very weak and broadly distributed. Surprisingly, after nuclear membrane breakdown (prometaphase), SUV39H1 staining displays a sudden accumulation in very bright small spots, which represent the centromeres (see Fig. 3, below). In metaphase, when the single chromosomes are individually visible, the stain is highly concentrated and reminiscent of the characteristic double-dotted centromeric pattern. However, very faint SUV39H1 staining is also detected at many non-centromeric sites, suggesting that SUV39H1 may have a broader, but much weaker affinity for the chromosomal arms. As soon as the two sister chromatids start to separate at the beginning of anaphase, the SUV39H1 stain is significantly reduced, although very low signals are still detected at the centromeres. In late anaphase, when the chromosomes have started to migrate to opposite poles, and in telophase, when the DNA starts to decondense...
again, the SUV39H1 stain remains at very low levels. We conclude that SUV39H1 displays a dynamic distribution at mitotic chromatin with a weak and rather dispersed staining at prophase, highly specific centromeric signals at prometaphase and metaphase, and again significantly reduced staining at the onset of anaphase.
Specific localisation of mitotic SUV39H1 with relation to HP1-like proteins

Since SUV39H1 and M31 (HP1β) are both members of a mammalian SU(VAR) protein complex and significantly colocalise in murine interphase cells (Aagaard et al., 1999), we next examined whether M31 would also overlap at mitotic chromatin in a similar fashion. Although a large portion of M31 is not bound to HeLa mitotic chromosomes (data not shown) (Minc et al., 1999), the chromatin-associated fraction does not appear to alter significantly during mitosis, M31 binds a much broader pericentric region than SUV39H1 and, in addition, also decorates several sites along the chromosomal arms (Fig. 2A, insert) (Wreggett et al., 1994; Furuta et al., 1997). From prometaphase throughout metaphase, as well as...

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**Fig. 4.** SUV39H1 associates with active centromeres. (A) Colcemid-arrested metaphase chromosomes of the dicentric human cell line 8828 were stained by immunofluorescence with α-Suv39h1 antibodies (pink) and by FISH with either a chromosome 14/22 α-satellite specific DNA probe (green, left panel), or a chromosome 15 α-satellite specific DNA probe (green, right panel). DNA was counterstained by DAPI (blue). The normal chromosomes 14 and 15 are marked by small arrows and the dicentric RT14q15q chromosome by big arrows and shown enlarged in the inserts. (B) Colcemid-arrested metaphase chromosomes of neocentromeric Brad cells were stained by immunofluorescence with α-Suv39h1 antibodies (pink) and by FISH with a chromosome 13 painting probe (green). DNA was counterstained with DAPI (blue). The normal chromosomes 13 are marked by small arrows. The rearranged chromosome 13 is marked by big arrow and shown enlarged in the insert.
in early anaphase, M31 remains associated with the chromosomes at comparable levels. Using double labelling, the same cells were analysed for the presence of SUV39H1, which specifically accumulates at centromeres only during prometa- and metaphase and largely disappears at the onset of anaphase.

Colocalisation of HP1α and SUV39H1 at metaphase chromosomes again indicated a relatively broad and persistent (data not shown) pericentric staining for HP1α, with additional signals in the chromosomal arms (Fig. 2B, insert) (Minc et al., 1999) – a pattern that resembles the mitotic distribution of M31. By contrast, SUV39H1 is almost exclusively detected as double-dotted signals at the centromeres. Thus, despite the significant heterochromatic overlap between M31 and SUV39H1 in interphase (Aagaard et al., 1999), the mitotic fractions of M31, and also of HP1α, display more extended spatial and temporal associations with mitotic chromatin with respect to the transient, centromere-specific localisation of SUV39H1.

**SUV39H1 transiently accumulates at the outer region of centromeres**

Double labelling experiments with human anti-centromere antibodies (hACA), which recognise CENP-A, CENP-B and CENP-C, confirm SUV39H1 to be centromeric. Consistent with previous observations (Aagaard et al., 1999), the SUV39H1 stain in metaphase is more towards the outer centromeric region as compared to the hACA stain (Fig. 3A, inserts, middle panels). Importantly however, whereas hACA signals do not change during meta- to anaphase transition, SUV39H1 staining is significantly reduced. In particular, at separated sisters in early anaphase, the SUV39H1 stain is almost absent from the chromosomes (Fig. 3A, inserts, lower panels). Double-labelling for cytoplasmic dynein, which redistributes to the distal face of the kinetochore at the beginning of metaphase (Wordeman et al., 1991), indicates colocalisation with SUV39H1 signals (Fig. 3B, inserts). These data define SUV39H1 as a component of the outer centromeric region that only transiently accumulates at centromeres during mitosis.

To further characterise the timing at which SUV39H1 associates and dissociates with centromeric positions of mitotic chromosomes, we also performed immunolocalisations for several well-characterised marker proteins (data not shown), including phosphorylated histone H3 (phosH3) (Hendzel et al., 1997; van Hooser et al., 1998), MAD2 (Li and Benezra, 1996) and CENP-E (Yen et al., 1992; Wood et al., 1997). The results of this multiple marker analysis indicated that SUV39H1 staining is suddenly enhanced at the centromere during prometaphase, after histone H3 phosphorylation, concomitantly with MAD2 and probably earlier than CENP-E. Moreover, SUV39H1 dissociates from the centromere at the meta- to anaphase transition, later than MAD2 and earlier than CENP-E. Together with the above colocalisations, these data reveal a novel spatial and temporal staining profile for SUV39H1 at mammalian centromeres.

**SUV39H1 associates with active centromeres**

The centromere-specific accumulation suggests a function for SUV39H1 at the centromere. To test this notion further, we analysed the localisation of SUV39H1 in a dicentric human cell line (8828) that carries a Robertsonian translocation of chromosomes 14 and 15 (RT14q15q). SUV39H1 was detected as a single pair of double dots on chromosomes in metaphase-arrested 8828 cells (Fig. 4A). To verify whether SUV39H1 was associated with the active centromere, which has been shown to be derived from chromosome 14 in RT14q15q (Sullivan and Schwartz, 1995), we subsequently subjected the spreads to FISH analysis using probes that are specific for either chromosome 14/22 or chromosome 15 α-satellite DNA. The chromosome 14/22 probe detects four signals (Fig. 4A, left panel), two on chromosomes 22, one on the normal, acrocentric chromosome 14 (small arrow) and one on the metacentric RT14q15q (big arrow). All four signals overlap with the SUV39H1 stain, indicating that SUV39H1 is present on the active centromere of the dicentric chromosome. The chromosome 15 specific probe gives rise to two signals (Fig. 4A, right panel), one on the normal, acrocentric chromosome 15 (small arrow) and the other one on the dicentric RT14q15q (big arrow). On the normal chromosome 15, the FISH signal and the SUV39H1 stain overlap, whereas on the RT14q15q the signals are just adjacent to each other (see insert), indicating that SUV39H1 does not localise to the inactive centromere.

To examine whether SUV39H1 requires α-sat DNA sequences for centromere association, we next analysed whether SUV39H1 would also recognise a neocentromere. For this experiment, we used a human cell line, which carries a chromosome 13 derived neocentromere (inv-dup13q21) lacking detectable α- and β-satellite DNA (Warburton et al., 1997). In this cell line, SUV39H1 also appears as a double dot on chromosomes in metaphase-arrested chromosomal spreads (Fig. 4B). To identify the neocentric chromosome, we subjected the spread to FISH analysis using a chromosome 13 specific painting probe. Because of its small size, the aberrant chromosome 13 (large arrow) can easily be distinguished from the corresponding normal chromosomes (small arrows). Importantly, SUV39H1 stain is detected on inv-dup13q21 (see insert), again indicating that SUV39H1 is a component of active centromeres.

**SUV39H1 is a phosphoprotein with mitosis-specific isoforms**

The transient centromeric accumulation of SUV39H1 could be caused either by altered protein stability or by post-translational modification. We therefore analysed whether the total protein levels of SUV39H1 may change during mitosis by synchronising HeLa cells in S phase with a double thymidine block. After release from the block, whole cell extracts were collected at timely intervals for 14 hours. As revealed by the accompanying FACS analysis, we examined the cell cycle from S phase through mitosis into the following G1 phase (Fig. 5A). Western blot analysis indicated that the total levels of SUV39H1 protein remain constant during this time course (Fig. 5B), as do the levels of the SUV39H1-interacting protein, M31. Thus, proteolytic degradation cannot account for the shift in SUV39H1 accumulation at centromeres during the cell cycle.

We next examined whether the redistribution of SUV39H1 could be correlated to post-translational modification. We prepared whole-cell extracts of exponentially growing and of nocodazole-arrested HeLa cells, and analysed possible shifts in SUV39H1 protein migration by two-dimensional gel electrophoresis, followed by western blotting. The α-Suv39h1
antibodies recognise a cluster of spots, of which the most basic represents unmodified SUV39H1, since it comigrates with in vitro translated SUV39H1 (Fig. 6, arrowhead). In exponentially growing cells, three more acidic spots and one very weak spot can be detected. In lysates from nocodazole-arrested cells, two additional, even more acidic, spots are observed (marked by arrows). To test whether these isoforms could be the consequence of phosphorylation, we treated the lysates with $\lambda$-phosphatase. In both the exponentially growing and the nocodazole-arrested cell lysates, the second most basic spot almost disappeared, and the most basic spot (arrowhead) was highly enriched. The pattern of the other spots in the exponentially growing cell lysates largely remained the same. In the nocodazole-arrested lysate, however, the two metaphase-specific spots completely disappeared, indicating that these two isoforms represent phosphorylated versions of SUV39H1. These results demonstrate that SUV39H1 becomes specifically phosphorylated during metaphase at at least two sites.

**SUV39H1 is preferably phosphorylated at serine residues**

To start characterising possible phosphorylation pathways, we next examined which amino acid residues in SUV39H1 would be preferred targets for phosphorylation. For this analysis, we transiently transfected into HeLa cells three flag-tagged constructs that encode full-length SUV39H1 (flag-SUV39H1; aa 3-412), the N terminus comprising the chromo domain (flag-Nchromo; aa 3-118) and an extended SET domain with an additional nuclear localisation signal (flagnls-SET; aa 161-412) (see Materials and Methods). Following in vivo labelling with ortho-phosphate and immunoprecipitation with $\alpha$-flag antibody beads, a similar enrichment of the ectopic proteins was confirmed by western blotting with $\alpha$-flag antibodies. The corresponding autoradiogram indicated that the full-length protein and the extended SET domain incorporated significant phosphate label, whereas the N-terminal portion of SUV39H1 displayed only a very weak signal (Fig. 7, middle panels).

Subsequent phospho-amino acid analysis of hydrolysed amino acids by 2-D thin layer chromatography revealed selective transfer of phosphate label to serine residues in the full-length and extended SET domain products (Fig. 7, bottom panels). Similarly, both phospho-serine and also, to a higher degree, phospho-threonine are detected at low amounts in hydrolysed amino acids from the flag-Nchromo peptide. By contrast, phosphorylated tyrosine is absent, even after long exposures of all three autoradiograms (data not shown). Combined with putative consensus site alignments (Kreegipuu et al., 1999; http://www.cbc.dtu.dk/ databases/PhosphBase), these data suggest the presence of five preferred serine phosphorylation sites within the SUV39H1 amino acid sequence, four of which cluster in the C-terminal tail and one resides at the beginning of the SET domain (Fig. 7, top diagram). In addition, two serine and two threonine phosphorylation sites are predicted in and around the chromo domain, but appear to be much less efficiently phosphorylated under our labelling conditions.

**DISCUSSION**

**SUV39H1 displays a novel spatial and temporal association pattern with human centromeres**

The centromere is a key structure to ensure correct chromosome transmission and is likely to be regulated by multiple pathways. Based on our comparative analysis, we show that SUV39H1 is a transient centromeric protein with a novel spatial and temporal association pattern (Figs 1-3). For example, in contrast to significant heterochromatic colocalisation in interphase (Aagaard et al., 1999), the mitotic fractions of M31 (HP1$\beta$), HP1$\alpha$ and SUV39H1 only partly overlap on prometa- and metaphase chromosomes. Whereas both HP1-like proteins broadly decorate pericentric heterochromatin and also associate with additional sites in the chromosomal arms (Fig. 2) (Minc et al., 1999), SUV39H1 specifically localises in a classical two-dotted pattern to the outer centromeric region, as indicated by
defining its position relative to CENPs and dynein (Fig. 3). Moreover, SUV39H1 accumulates at human centromeres in prometaphase after histone H3 phosphorylation, together with MAD2, but probably before CENP-E, and it dissociates at the meta- to anaphase transition after MAD2 and before CENP-E (data not shown).

The INCENPs belong to a class of ‘passenger proteins’ that also dissociate from chromatin at the meta- to anaphase transition. In addition, INCENP has been shown to interact with HP1α, which apparently modulates INCENP targeting to the mitotic spindle (Ainsztein et al., 1998). However, whereas the INCENPs are located between sister chromatids in the inner centromeric regions during early metaphase and translocate off chromosomes at late metaphase (Earnshaw and Cooke, 1991), the SUV39H1 staining pattern is clearly distinct. SUV39H1 localises to the outer regions of the centromere throughout metaphase (see above) (Aagaard et al., 1999) and accumulates at centromeres already at prometaphase. Together, these results define a novel distribution pattern for SUV39H1 at human centromeres, which suggests a different function in centromere-dependent chromatin organisation (see below).

**SUV39H1 is a phosphoprotein**

On 2-D gel electrophoresis, SUV39H1 runs as a cluster of a total of seven spots, two of which represent mitosis-specific phosphorylated isoforms (Fig. 6). Phospho-amino acid analysis indicates that SUV39H1 is preferably phosphorylated at serine and also, to a lesser extent, on threonine residues but lacks detectable tyrosine phosphorylation (Fig. 7). Considering these data, consensus site alignments (Kreegipuu, 1999) suggest seven putative serine and two putative threonine phosphorylation sites within the SUV39H1 amino acid sequence, none of which are currently mapped experimentally.

Based on these predictions, four serine phosphorylation sites cluster in the C-terminal tail (373-DMESTRMDSNF-GLAGLPSPKKVREIECKTGESCRKYLF*), one serine phosphorylation site is present at the beginning of the SET domain (265-RKNSFVM), and two serine (33-LGISKRN; 69-DSESTWE) and two threonine (68-SESTWEP; 103-RKTPRHI) phosphorylation sites are localised in the middle of or immediately adjacent to the chromo domain (see top diagram and legend of Fig. 7). Since SET domains can be distinguished by their C-terminal extensions (Jenuwein et al., 1998), the conspicuous clustering of potential serine phosphorylation sites may reveal the C-terminal tail as a novel protein module to regulate SUV39H1-specific functions.

A subset of the SUV39H1 phosphorylation sites is cell-cycle regulated, inferring cyclin dependent kinases (Cdk) as possible
candidates for mitotic phosphorylation, particularly since p34cdc2 has been shown to be present at paired kinetochores during metaphase (Rattner et al., 1991). Only one putative Cdc2 phosphorylation site (LPGSPKK) is predicted in the C-terminal tail; this site is not conserved throughout evolution (Jenuwein et al., 1998). Recently, HP1 association with heterochromatin was shown to be regulated by casein kinase II (CKII) dependent phosphorylation of three sites, one site preceding the chromo domain and two other sites residing at the very end of the chromo shadow domain (Zhao and Eissenberg, 1999). Although these sites are not conserved in the chromo domain of SUV39H1, there is a CKII consensus site (DSESTWE) in the middle of the chromo domain. Moreover, phosphorylation of the N-terminal portion of SUV39H1 including the chromo domain is highly underrepresented with respect to the extended SET domain (see Fig. 7) – a result that may indicate a more cell cycle restricted phosphorylation of the chromo domain-associated target residues.

Phosphorylation-dependent modification of SUV39H1 could also be controlled by removal/protection of phosphates, since the SET domains of several proteins have been shown to interact with the myotubularin family of dual specificity phosphatases (dsPTPases) and their inhibitor Sbf1 (SET binding factor 1) (Cui et al., 1998). Finally, the preference for serine phosphorylation would make SUV39H1 also a possible substrate for aurora-like kinases (Glover et al., 1995; Biggins et al., 1999) and their antagonistic phosphatases (Axton et al., 1990; Sassoon et al., 1999), particularly since deregulated SUV39H1 interferes with chromosome segregation and
induces a high incidence of multinucleated cells (M. Melcher and T. J., in preparation). Intriguingly, protein phosphatase 1 has been classified as a Su(var) gene in Drosophila (Baksa et al., 1993).

A regulatory role(s) for SUV39H1 at the mammalian centromere?

The dynamic association with mitotic chromatin suggests several possible functions for SUV39H1. For example, SUV39H1 accumulates at centromeres during prometaphase, but dissociates from the centromere at the meta- to anaphase transition (Figs 1-3), consistent with a possible role in sister chromatid cohesion or segregation. Although a cohesin complex has been identified in S. cerevisiae (Uhlmann et al., 1999), in multicellular eukaryotes, the vast majority of cohesin homologues are displaced from the chromosomes at the onset of mitosis, long before sister chromatid separation is initiated (Losada et al., 1998). These results have given rise to interpretations that there may be two distinct cohesin complexes in multicellular eukaryotes, regulating cohesion in the chromosomal arms and at the centromere. At this stage of the analysis, we cannot exclude a possible role for SUV39H1 in cohesion, particularly for a centromere-dependent mechanism. By contrast, SUV39H1 is unlikely to be part of known mitotic checkpoint pathways, since (1) SUV39H1 stays on the chromosomes longer than MAD2 even after they have aligned properly (data not shown), and (2) because colcemid treatment does not alter SUV39H1 localisation (Aagaard et al., 1999). In addition, although the MAD/BUB pathway is highly conserved from yeast to man, we have failed to identify a SUV39H1-related gene in S. cerevisiae.

The sudden accumulation of SUV39H1 at centromeric positions during prometaphase partly coincides with the spatial and temporal distribution of the mitotic condensation marker phosH3 (Hendzel et al., 1997; van Hooser et al., 1998). Although H3 phosphorylation precedes and persists SUV39H1 phosH3 (Hendzel et al., 1997; van Hooser et al., 1998). Although SUV39H1 interferes with the G2-specific definition of phosH3-positive foci (M. Melcher et al., unpublished). As prometaphase is characterised by increased chromosome organisations, SUV39H1 could, therefore, play a role in centromere-directed alignments of condensing chromosomes, as they move to the metaphase plate. In agreement with such a function, SUV39H1 selectively associates with active centromeres (Fig. 4). Since in addition SUV39H1 is localised to the outer centromeric region (Fig. 3) (Aagaard et al., 1999), it may be involved in microtubule attachment – a notion further supported by the genetic interaction between microtubule mutants and Ctr4 in S. pombe (Ekwall et al., 1996). Based on its intriguing distribution and modification pattern described here, we propose that SUV39H1 may even play a regulatory role(s) in defining a specific higher order chromatin structure at mammalian centromeres.

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