

Transfer of Ser7 Phosphorylated CENP-A from Centromere to Midbody During Mitosis in MCF-7 Cells

(Ser7 phosphorylated CENP-A / centromere / midbody / cytokinesis)

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Abstract. Serine 7 of centromere protein A (CENP-A) is a very important mitosis-specific phosphorylation site. In this study, we demonstrate the subcellular distribution of Ser7 phosphorylated CENP-A during mitosis in MCF-7 cells. The Ser7 phosphorylation of CENP-A was observed beginning at prophase at centromeres. Upon progression of mitosis, the fluorescence signals emerged in the central region of the metaphase plate and were maintained until anaphase at centromeres. At late anaphase, the fluorescence signals moved to the midzone gradually and transferred from the centromere to the midbody completely at telophase. They were compacted into the centre of the midbody in a thin cylinder consisting of a sandglass-like “mitotic machine” with microtubules and condensed chromosome. We also found that Ser10 phosphorylated H3 and Thr11 phosphorylated H3 were co-localized at the midbody in two bell-like symmetrical bodies with Ser7 phosphorylated CENP-A during the terminal stage of cytokinesis. Midbody isolation and immunoblotting experiments also indicated that Ser7 phosphorylated CENP-A are components of the midbody. These findings suggest that Ser7 phosphorylated CENP-A acts

as a chromosomal passenger protein and may play an important role in cytokinesis.

Introduction

Eukaryotic cells must possess mechanisms for condensing and decondensing chromatin. Chromatin condensation is particularly evident during mitosis and cell death induced by apoptosis, whereas chromatin decondensation is necessary for replication, repair, recombination and transcription. Post-translational modification of histone tails plays a critical role in the dynamic condensation/decondensation that occurs during the cell cycle (Prigent and Dimitrov, 2003). One of the post-translational modifications is phosphorylation, which is believed to play a direct role in mitosis, cell death, repair, replication and recombination (Ito, 2007).

Histone H3 phosphorylation is a particularly interesting epigenetic mark involved in chromatin remodelling and gene expression (Delcuve et al., 2009). There is a precise spatial and temporal correlation between H3 phosphorylation and initial stages of chromatin condensation. An increase in histone H3 Ser10 phosphorylation is a well-known hallmark for mitosis and meiosis in various eukaryotic organisms. Mitotic phosphorylation of H3 at Ser10 initiates in pericentromeric heterochromatin in late G2 and spreads throughout the condensing chromatin (Hendzel et al., 1997). Recent studies have shown that H3 is also phosphorylated at Ser28, Thr11, Thr3, and H3.3 Ser31 during mitosis (Goto et al., 1999; Wei et al., 1999; Preuss et al., 2003; Polioudaki et al., 2004; Hake et al., 2005). Ser10, Ser28 and Thr3 phosphorylated H3 are excluded from centromeric chromatin in mammalian cells; Thr11 phosphorylated H3 is not distributed over the whole chromosomes but specially localizes to centromeric chromatin (Hendzel et al., 1997; Goto et al., 1999, 2002; Preuss et al., 2003; Zhou et al., 2008).

The centromere is a specialized chromosomal structure that regulates faithful chromosome segregation during cell division. In all eukaryotes, the centromere-specific histone H3 variant CENP-A replaces canonical H3 within nucleosomes at centric chromatin (Dalal, 2009;

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Abbreviations: CENP-A – centromere protein A, DMEM – Dulbecco's modified Eagle's medium, ECL – enhanced chemiluminescence, FBS – foetal bovine serum, FITC – fluorescein isothiocyanate, H3 – histone 3, HRP – horseradish peroxidase, INCENP – inner centromere protein, pCENP-A – phosphorylated CENP-A, pH3 – phosphorylated histone 3, Ser7 – serine 7, Thr11 – threonine 11.

Torras-Llort et al., 2009). However, extended chromatin fibre technique showed that no centromeric chromatin fibres contained only CENP-A and no H3. Along centromeric chromatin fibres, CENP-A appeared to alternate with H3 (Blower et al., 2002). The N-terminal tail of CENP-A shares a mitotic phosphorylation motif with canonical histone H3 and is phosphorylated at Ser7 in mitotic cells (Zeitlin et al., 2001a; Kunitoku et al., 2003; Slattery et al., 2008). Thus, it has been proposed that Ser7 phosphorylated CENP-A may participate in the maturation or function of the active kinetochore (Smith, 2002; Preuss et al., 2003).

Interestingly, the Ser10 phosphorylated H3 is associated with the mitotic spindle through anaphase and remains within the midzone and midbody until completion of cytokinesis as a chromosomal passenger (Li et al., 2005; Song et al., 2007). The midbody is a microtubule array within the intercellular bridge that connects two daughter cells. It is a derivative of the midzone that forms by compression of the furrow during ingression (Eggert et al., 2006). In addition to the microtubule bundle, the midbody contains a number of spindle and chromosome-derived proteins packed into the phase-dense 'Flemming body' at the centre of the intercellular bridge. These components may actively participate in the scission, the final step of cytokinesis (Zeitlin and Sullivan, 2001).

In the present study, we investigated the dynamic distribution of Ser7 phosphorylated CENP-A during mitosis. The results showed that Ser7 phosphorylated CENP-A departed from the centromere and transferred to midbody during cytokinesis. The midbody localization indicates that they may play a role in the process of the separation of nascent daughter cells. To identify whether they are components of the midbody, we isolated midbodies from mitotically-arrested cells and analysed their protein components by Western blotting. The results revealed that Ser7 phosphorylated CENP-A are components of the midbody. All the evidences suggest that Ser7 phosphorylated CENP-A have a functional significance during cytokinesis.

Material and Methods

Cell lines and culture conditions

MCF-7 (human breast cancer) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, BRL, Carlsbad, CA), supplemented with 10% foetal bovine serum (FBS, Gibco) and 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a humidified 37 °C incubator with a 5% CO₂ atmosphere.

Immunofluorescence and confocal laser microscopy

MCF-7 cells were cultured on glass cover slips for two days. The cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) at room temperature for 10 min. After

washing in PBS, the cells were permeabilized in 0.2% Triton-X 100 in PBS for 10 min, washed once in PBS containing 0.1% Tween 20 (PBS-T), and blocked with 2% BSA in PBS for 45 min at room temperature. The specimens were washed once in PBS-T for 5 min and subsequently incubated with the rabbit anti-phospho-CENP-A Ser7 antibody (Millipore, Billerica, MA) diluted at 1:100 and mouse anti- α -tubulin antibody (Santa Cruz, Dallas, TX) diluted at 1:100 in a humid chamber for 1 h at 37 °C or overnight at 4 °C and then washed three times in PBS-T for 5 min. As secondary antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Santa Cruz) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Santa Cruz) were diluted at 1:100 with 1% BSA in PBS for 45 min at 37 °C. After final rinse in PBS, the cells were counterstained with DAPI and mounted onto slides. Three colour images were acquired using Leica TCS SP5 laser scanning confocal microscope with a 40 \times or 63 \times oil immersion objective (Leica Microsystems, Wetzlar, Germany).

Co-detection of Ser10 or Thr11 phosphorylated H3 and Ser7 phosphorylated CENP-A

For co-detection of Ser10 or Thr11 phosphorylated H3 and Ser7 phosphorylated CENP-A with rabbit antisera, we performed a successive labelling method with an additional blocking step to facilitate co-detection without cross-reaction (Zeitlin et al., 2001a). The cells were first incubated with anti-phospho-CENP-A Ser7 antibody diluted at 1:100. After washing three times in PBS-T, the secondary antibody (goat anti-rabbit TRITC, Santa Cruz) was added at a dilution of 1:100. After washing three times, cells were blocked with 5% non-fat dry milk in PBS-T for 45 min at room temperature. Then anti-phospho-histone H3 Ser10 or Thr11 antibody was used to perform the second staining at a dilution of 1:100 in 1% BSA in PBS-T for 1 h at 37 °C. The cover slips were washed again, and the secondary antibody (goat anti-rabbit FITC, Santa Cruz) was used at 1:100 dilution. Finally, the cover slips were washed again with PBS before mounting.

Cell synchronization and midbody isolation

Synchronization of the cell cycle was achieved using successive thymidine and nocodazole blocks (Whitfield et al., 2000). MCF-7 cells were grown to 40–60% confluence and synchronized by arresting them at the boundary between G1 and S phase by adding 2.5 mM thymidine for 18 h. Cells were washed free of thymidine with sterile PBS and returned to fresh medium for 3 h, and then arrested in metaphase with 100 ng/ml nocodazole. After 12 h, the cells were again released from the nocodazole block with two washes of sterile PBS and allowed to progress into telophase. By 35 min into the recovery period the mitotically-arrested cells had furrowed and formed midbodies. The midbodies were iso-

lated from MCF-7 cells according to the procedure described previously (Mullins and McIntosh, 1982).

SDS-PAGE and Western blotting

The samples of isolated midbodies were resolved by 15% SDS-PAGE. The proteins were transferred onto PVDF membranes (Millipore). The blots were blocked with 5% non-fat dry milk (Amresco, Solon, OH) in TBS (pH 7.4) containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature and subsequently incubated with primary antibodies for 1 h or overnight at 4 °C. Membranes were washed three times in TBS-T and detected with secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h. After three washes in TBS-T, the specific proteins were visualized using enhanced chemiluminescence (ECL) detection reagent (Millipore) and exposed to BioMax film (Kodak, Radnor, PA).

Results

Ser7 pCENP-A acted like chromosomal passenger proteins during mitosis

Three colour fluorescent labelling techniques were used to exhibit the dynamic distribution of Ser7 phosphorylated CENP-A at different phases of mitosis in MCF-7 cells. Cells were counterstained with DAPI to visualize chromatin (blue) to estimate mitotic stages. Also, the α -tubulin in the cell was labelled with TRITC (red) to show the cell scaffold and movement. The results showed that CENP-A Ser7 phosphorylation initially appeared at centromere with chromosome condensation concurrently at prophase (Fig. 1a, b). At metaphase, the green fluorescence signal of Ser7 pCENP-A settled at the equatorial plate of the mitotic spindle (Fig. 2c). At anaphase, the signal of Ser7 pCENP-A transferred from centromere to the spindle midzone (Fig. 2d). At telophase, the green signals of Ser7 pCENP-A were concentrated into the centre in a thin cylinder consisting of the sandglass-like “mitotic machine” with microtubules and condensed chromosome (Fig. 1e, f). Finally, the Ser7 pCENP-A were transformed into two bell-like structures localized in the centre of two arms of the midbody (Fig. 1g). It is very interesting that the distribution of Ser7 pCENP-A during the terminal stages of cytokinesis was similar to that characteristic of chromosomal passenger protein.

Ser7 pCENP-A co-localized with Thr11 or Ser10 pH3 at the midbody

Previous data have proved that Ser10 pH3 and Thr11 pH3 can also locate at midbody during cytokinesis. In order to find out the relationship between phosphorylated H3 and phosphorylated CENP-A, we performed co-detection of the distribution pattern of Ser10 or Thr11 phosphorylated H3 and Ser7 phosphorylated CENP-A in MCF-7 cells by immunofluorescence. The results showed that Ser10 pH3 or Thr11 pH3 and Ser7 pCENP-A also participate in the midbody assembly. Both Ser10

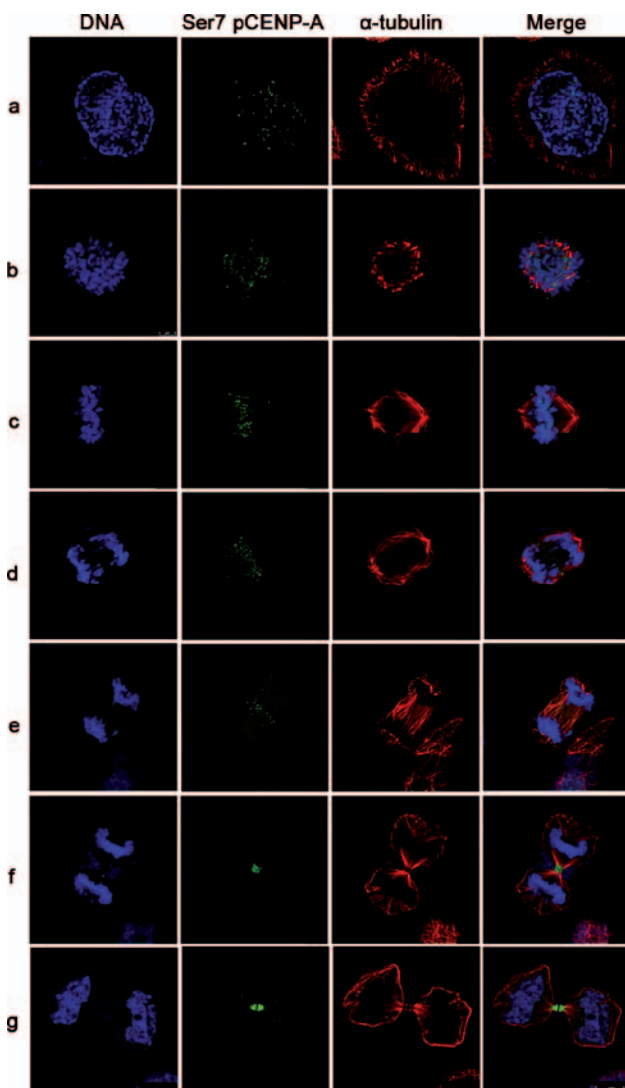


Fig. 1. Dynamic distribution of Ser7 pCENP-A in MCF-7 cells during mitosis. The Ser7 pCENP-A was labelled with FITC (green); α -tubulin and DNA were labelled with TRITC (red) and DAPI (blue), respectively. a, b: prophase; c: metaphase; d: anaphase; e: telophase; f, g: cytokinesis. The bar represents 10 μ m.

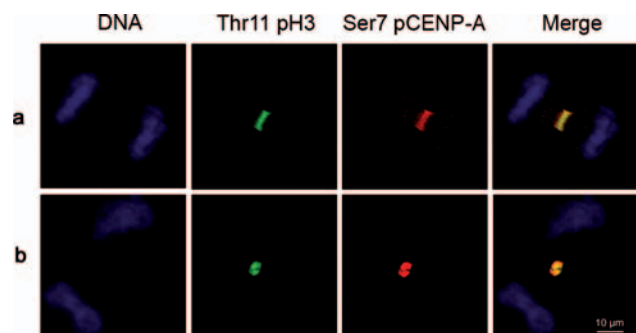


Fig. 2. Co-detection of Thr11 pH3 and Ser7 pCENP-A at cytokinesis in MCF-7 cells. Thr11 pH3 were labelled with FITC (green), Ser7 pCENP-A and DNA were labelled with TRITC (red) and DAPI (blue), respectively. a, b: cytokinesis. The bar represents 10 μ m.

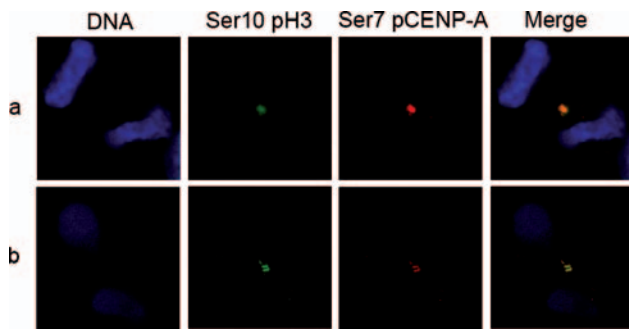


Fig. 3. Co-detection of Ser10 pH3 and Ser7 pCENP-A at cytokinesis in MCF-7 cells. Ser10 pH3 were labelled with FITC (green), Ser7 pCENP-A and DNA were labelled with TRITC (red) and DAPI (blue), respectively. a, b: cytokinesis. The bar represents 10 μ m.

pH3 or Thr11 pH3 and Ser7 pCENP-A were compacted into the centre of the midbody in a thin cylinder (Fig. 2a and Fig. 3a). During the terminal stages of cytokinesis, Ser10 pH3 or Thr11 pH3 and Ser7 pCENP-A co-localized at the midbody in two bell-like symmetrical bodies to construct the functional midbody (Fig. 2b and Fig. 3b). Our results showed that Ser7 phosphorylated CENP-A have absolutely the same location as phosphorylated H3, which may imply that they play the same role in cytokinesis.

Ser7 pCENP-A are components of the midbody

SDS-PAGE and Western blotting analysis were performed to examine the phosphorylated CENP-A and phosphorylated H3 at the midbody. The sample of midbodies isolated from the mitotically-arrested cells and interphase cells (as negative control) of MCF-7 were separated into a group of bands by 15% SDS-PAGE (data not shown). As is well known, α -tubulin is one of the main components of the midbody. At the same time, previous data and our data have indicated that aurora B and Ser10 pH3 are components of the midbody. Aurora B and α -tubulin thus served as positive controls in Fig. 4. Our results showed that Ser7 pCENP-A and Thr11 pH3 could both be detected in the midbody isolated from the mitotically-arrested cells but not in the midbody isolated from interphase cells (Fig. 4). The results further confirmed that Ser7 pCENP-A and Thr11 pH3 are components of the midbody.

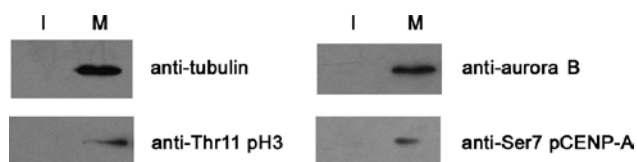


Fig. 4. Isolated midbodies were analysed by SDS-PAGE and Western blotting. Lane I – midbody sample from interphase cells (negative control); lane M – midbody sample from mitotically-arrested cells. The antibodies used were anti- α -tubulin, anti-aurora B, anti-Thr11 pH3 and anti-Ser7 pCENP-A.

Discussion

Phosphorylation of histone H3 is a hallmark event in mitosis and is associated with chromosome condensation (Garcia et al., 2005). There are four characterized phosphorylated residues within the H3 N-terminal tail during mitosis: Thr3, Ser10, Thr11, and Ser28. All of these phospho-H3 marks are present during mitosis, suggesting a possible role in chromatin condensation (Perez-Cadahia et al., 2009). However, the temporal and spatial distribution of these sites in phosphorylated histone H3 differed from each other during mitosis.

The centromere is a specialized locus on chromosomes that mediates attachment of microtubules during mitosis via a large multiprotein complex called the kinetochore. Centromere protein A (CENP-A) is an essential histone H3-like kinetochore protein incorporated specifically at active centromeres. CENP-A is phosphorylated at Ser7 by aurora kinase and plays an unexpected role in completion of cytokinesis (Zeitlin et al., 2001b; Kunitoku et al., 2003). Dominant negative phosphorylation site mutants of CENP-A result in a delay at the terminal stage of cytokinesis (Zeitlin et al., 2001b). We observed that dynamic distribution of Ser7 pCENP-A during the terminal stages of mitosis is similar to the characteristic of chromosomal passenger protein. During the final step of cytokinesis, Ser10 or Thr11 phosphorylated H3 overlapped with Ser7 phosphorylated CENP-A, and co-localized at the midbody in two bell-like symmetrical bodies to support cytokinesis. These results suggest that Ser7 phosphorylated CENP-A not only triggered chromatin condensation, but also released themselves from the chromosome to join in nuclear division by construction of the sandglass-like “mitotic machine”.

Chromosomal passenger proteins associate with chromosomes early in mitosis and transfer to the spindle at ana/telophase. They are present in cells as a complex with at least four members: aurora B, which is responsible for mitotic histone H3 phosphorylation, inner centromere protein (INCENP), survivin and borealin. Aurora B can bind to survivin and the C-terminal region of INCENP, respectively, and co-localizes with both proteins to the centromeres, midzone and midbody (Terada, 2001; Vagnarelli and Earnshaw, 2004). Recently, it has been proposed that Ser10 phosphorylated H3 is involved in cytokinesis as a chromosomal passenger. Co-immunoprecipitation indicated that the Ser10 phosphorylated H3 was associated with aurora B, and both proteins were compacted into a complex with a special ternary structure located in the centre of the midbody. When the level of the Ser10 phosphorylated H3 was reduced by RNA interference, the cells formed an aberrant midbody and could not complete cytokinesis successfully (Song et al., 2007). These chromosomal passenger proteins participate in mitosis and play key roles in cytokinesis. The evidence supports the idea that nuclear division and cytokinesis of mammalian cells were coupled with chromosomal passenger protein phosphorylation.

Midbody, a transient organelle-like structure, is known as central for abscission and is indispensable for termination of cytokinesis. Cytokinesis, an event common to all eukaryote organisms, is the process by which a dividing cell splits into two and is essential for cell division. Failure of cytokinesis can cause cell death or lead to genome amplification, which is characteristic of many cancers (Chen et al., 2009). Our midbody isolation and immunoblotting experiments demonstrated that Thr11 phosphorylated H3 and Ser7 phosphorylated CENP-A are components of the midbody, which suggests that phosphorylation of H3 and CENP-A may have important roles during cytokinesis.

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