H1.X with different properties from other linker histones is required for mitotic progression

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Abstract We report here the characterization of H1.X, a human histone H1 subtype. We demonstrate that H1.X accumulates in the nucleolus during interphase and is distributed at the chromosome periphery during mitosis. In addition, the results of fluorescence recovery after photobleaching indicate that the exchange of H1.X on and off chromatin is faster than that of the other H1 subtypes. Furthermore, RNA interference experiments reveal that H1.X is required for chromosome alignment and segregation. Our results suggest that H1.X has important functions in mitotic progression, which are different from those of the other H1 subtypes.

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1. Introduction

Histone H1 stabilizes chromatin structure by binding close to the entry–exit sites of linker DNA [1] and is thought to be important for chromatin condensation and the chromatin higher structure. In fact, depletion of histone H1 leads to structural and functional defects of chromosomes [2,3] and affects proper embryonic development [4]. In mammals, 10 H1 subtypes have been reported to date, i.e., H1.1–H1.5 [5,6], H1.6 [7], H1.t [8], H1.Foo [9], HILS1 [10] and H1.X [11].

Our previous studies of human metaphase chromosomes indicated that H1.X is a structural component of chromosomes [12,13]. H1.X expression is commonly detected throughout human tissues [11]. Its amino acid sequence does not show high similarities with the other somatic histone H1 subtypes (~30%), although it does possess some of the characteristic features of linker histones [14]. In addition, H1.X is partially associated with nucleosomes and enriched in micrococcal nuclease-resistant chromatin [15].

In the present study, we analyzed the function of H1.X in mitosis, since the biological functions of H1.X have not yet been elucidated. We demonstrate by fluorescence recovery after photobleaching (FRAP) and RNA interference (RNAi) analyses that H1.X has distinct properties from the other H1 subtypes that are essential for mitotic progression.

2. Materials and methods

2.1. Cells and transfection

HeLa cells were grown in Dulbecco’s modified Eagle’s medium (GIBCO BRL) supplemented with 10% fetal bovine serum (Equitech-Bio Inc.). For construction of a cell line stably expressing GFP-H1.X, a cDNA of H1.X was inserted into a pEGFP-C1 vector (Clontech), and the resulting plasmid encoding H1.X-GFP was transfected into HeLa cells using the FuGene6 reagent (Roche). A GFP-tagged expression vector for H1.2 visualization was constructed by integration of a human H1.2 cDNA into pEGFP-C1.

2.2. Antibodies

The primary antibodies used were: anti-H1.X rabbit polyclonal (Abcam), 1:200 dilution; anti-nucleolin mouse monoclonal (Upstate Biotechnology), 1:50 dilution; anti-α-tubulin mouse monoclonal (Calbiochem), 1:100 dilution; anti-γ-tubulin rabbit polyclonal (Sigma), 1:2000 dilution; anti-Bub1 mouse monoclonal (MBL), 1:20 dilution; anti-BubR1 mouse monoclonal (BD Transduction Laboratories), 1:1000 dilution; anti-MAP2 rabbit polyclonal (Covance), 1:100 dilution; and CREST (Cortex Biochem), 1:1000 dilution.

2.3. RNAi

A small interfering RNA (siRNA) duplex for H1.X (5’-CCAA-GAAGGUUCCGUGGUUTT-3’) was chemically synthesized and HeLa cells were transfected with 120 nM of the siRNA duplex using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. A control siRNA duplex (5’-UUUCUGAGAAGCUGUACUGU-3’: Qiagen) was used for control transfections. Cells were collected at 48 h after transfection and used for further analysis.

2.4. Immunofluorescence microscopy

HeLa cells grown on coverslips were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at 37 °C, and then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After incubation with 1% BSA-PBS for 15 min, the cells were incubated with a primary antibody for 1 h at room temperature, followed by incubation with an appropriate secondary antibody for 1 h. The secondary antibodies (labeled with Alexa 488 or TRITC) were used at a dilution of 1:200. After washing, the cells were stained with 1 µg/ml 4’,6-diamidino-2-phenylindole (DAPI) and observed under a microscope (Olympus) and analyzed using the Delta vision software (Applied Precision).

Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; GFP, green fluorescent protein; FRAP, fluorescence recovery after photobleaching; DAPI, 4’, 6-diamidino-2-phenylindole; NEBD, nuclear envelope breakdown

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Metaphase chromosome spreads were prepared as described previously [12].

2.5. FRAP analysis
FRAP analysis was performed using an IX-71 inverted microscope (Olympus) equipped with an FV300 scanning unit. First, a single image was captured by the 488 nm-line of a 10 mW-argon laser (set to 1%) and then a selected area (2.3 × 2.3 μm²) was bleached by the 488 nm-line of the argon laser (set to 40%). After 3 s of photobleaching, images were captured using the same scanning settings as above at intervals of 1.1 s (GFP-H1.X) or 20 s (GFP-H1.2). The relative intensities were calculated as follows:

Relative intensity = \( \frac{I_t}{W_t} \) / \( \frac{I_0}{W_0} \)

![Fig. 1. Mitotic defects in H1.X-depleted cells and localization of H1.X during the cell cycle.](image-url)
where $I_0$ is the average intensity in the region of interest before photobleaching, $I_t$ is the average intensity in the region of interest at time point $t$, $W_0$ is the average intensity in both photobleached and non-photobleached regions before photobleaching and $W_t$ is the average intensity in both photobleached and non-photobleached regions at time point $t$. The $t_{1/2}$ value was defined as the time required to reach half-maximum recovery and calculated from the corrected recovery curves.

3. Results

3.1. H1.X is required for accurate mitotic progression

To examine the biological function of H1.X, we performed RNAi of H1.X. Immunoblot and immunofluorescence analyses revealed that the abundance of H1.X was greatly decreased (Fig. 1A, E and G). The specificity of the anti-H1.X antibody used in this study was confirmed by immunoblotting (Fig. S1A). Furthermore, H1.X depletion had no influence on the expression of H1.2 (Fig. S1F). As shown in Fig. 1B, the mitotic index was elevated to 8.0 ± 1.2% in H1.X RNAi cells, compared to 4.9 ± 1.0% in control RNAi cells ($p < 0.05$). In H1.X-depleted cells, chromosome condensation occurred normally, and there was no influence on the localizations of known chromosomal proteins, namely hCAPE, topoisomerase IIa, CENPA, CENPE and CENPF (Fig. S2). However, chromosome alignment at the metaphase plate was impaired by H1.X depletion (Fig. 1C and G). We categorized the defects in chromosome alignment into the following two groups: misalignment chromosomes (1–10 chromosomes were not aligned at the metaphase plate, whereas the majority of the chromosomes were aligned) and non-alignment chromosomes (>10 chromosomes were not aligned at the metaphase plate or chromosomes were scattered throughout the cytoplasm). The misalignment and non-alignment chromosomes were detected in 16.1% and 6.5% of H1.X-depleted mitotic cells, respectively, compared to the corresponding values of 2.1% and 0.2% of control cells. In addition, lagging chromosomes were observed at anaphase in 1.4% of H1.X-depleted mitotic cells, compared to 0.2% of control cells. These defects in chromosome dynamics and mitotic progression were also observed in living cells (Fig. S3). The above phenotypes of H1.X-depleted cells were found to be reproducible when similar RNAi experiments were carried out using another siRNA sequence for H1.X (Fig. S1B-E).

3.2. Histone H1.X is localized to nucleoli and mitotic chromosomes

To examine the intracellular localization of H1.X, we performed immunostaining with an anti-H1.X antibody (Fig. 1D). H1.X was predominantly localized in the nucleoli of interphase cells. The precise nucleolar localization of H1.X was confirmed by counterstaining of the nucleolar proteins nucleolin and fibrillarin. The nucleolar localization pattern of H1.X differed from those of nucleolin and fibrillarin (Figs. 1E and S1G). The signal intensity of H1.X in the nucleoli was significantly decreased following depletion of H1.X.

During mitosis, H1.X was distributed on chromosomes, especially at the chromosome periphery (Fig. 1F). In H1.X RNAi cells, the H1.X signals around the chromosomes disappeared and chromosome misalignment was observed (Fig. 1G).

Fig. 2. H1.X dynamics during the cell cycle. (A) Comparison of the localization patterns of GFP-H1.X and GFP-H1.2 in living HeLa cells throughout the cell cycle. Bar, 5 μm. (B) FRAP recovery curves for GFP-H1.X at interphase (blue) and anaphase (orange). The FRAP recovery curve for GFP-H1.2 at interphase (red) is also indicated. The plot shows the relative recovery of fluorescence in the photobleached region versus time.
3.3. H1.X is dynamically associated with chromatin

To observe the dynamics of H1.X in living cells, we developed a stable HeLa cell line that constitutively expressed H1.X fused to enhance green fluorescent protein (EGFP) at its N-terminus. GFP-H1.X was mainly localized in nucleoli at interphase and chromosomes during mitosis (Fig. 2A). The localization pattern of H1.X differed from that of the typical linker histone H1.2. At interphase, histone H1.2 was mainly localized in the nucleoplasm (Fig. 2A). During mitosis, GFP-H1.2 signals were detected in the chromosomes, and not in the cytoplasm.

To quantitatively measure the binding kinetics of H1.X to unperturbed chromatin in living cells, we performed a FRAP analysis. As shown in Fig. 2B, the fluorescence recovery rate of H1.X ($t_{1/2} = 4.7 \pm 1.9$ s) was significantly faster than that of H1.2 ($t_{1/2} = 126.9 \pm 24.1$ s). Consistent with these findings, endogenous H1.X was dissociated from chromatin by a lower concentration of NaCl than H1.2 (data not shown). Thus, the interaction between H1.X and chromatin is considerably weaker than that between chromatin and H1.2. We found that the exchange rate of H1.X remained unchanged between interphase and anaphase. This observation was in strong contrast to the property of H1.2, which shows dynamic changes in its chromatin binding persistency during the cell cycle [16].

3.4. Depletion of H1.X induces aberrant spindle formation

The prolonged mitosis observed in H1.X-depleted cells (Fig. S3) suggests that the spindle checkpoint is activated by the depletion of H1.X. Actually, spindle checkpoint proteins, including Bub1, BubR1 and Mad2, were accumulated at the kinetochores of misaligned chromosomes (Fig. 3A), while the expression levels of cell cycle-related proteins required for the transition from metaphase to anaphase, namely CDC20, CDC27 and Plk1, were the same as those in control cells (Fig. S4B and C). Furthermore, double knockdown of H1.X with Aurora B, one of the spindle checkpoint proteins,
creased the mitotic index from 8.0% to 6.1% in H1.X-depleted cells (Fig. 1B). These results indicate that the mitotic arrest caused by H1.X depletion was due to spindle checkpoint activation rather than failure of metaphase exit.

To further examine whether H1.X is required for accurate attachment of microtubules to kinetochores, we analyzed kinetochore fiber formation in RNAi cells. In control cells, end-on attachments of microtubules to the kinetochores from the opposite spindle poles were clearly observed (Fig. 3B). In contrast, syntelic attachments were detected in misaligned chromosomes after H1.X depletion. In addition, three types of defect in spindle morphology were observed (Fig. 3C and D). In multipolar cells, more than three spindle poles were detected. This phenotype was observed in 1.8% of H1.X-depleted mitotic cells, compared to 0.4% of control cells. The most frequently observed defect in spindle morphology was spindle disorganization. In this case, the mitotic spindle appeared less focused and the symmetric formation of the spindle was impaired. Spindle disorganization was observed in 3.9% of H1.X-depleted mitotic cells, compared to 0.2% of control cells. The inter-centromosome distance was increased in 1.7% of mitotic H1.X-depleted cells. These results strongly suggest that H1.X is required for accurate mitotic spindle formation.

4. Discussion

We have demonstrated that H1.X is localized in nucleoli by immunostaining with an anti-H1.X antibody and observation of GFP-tagged H1.X (Figs. 1 and 2), whereas a somatic H1 subtype, H1.2, is not localized in nucleoli. The alternative localization of H1.X implies a relationship with nucleolar functions, such as rDNA transcription and rRNA maturation. However, depletion of H1.X did not affect the localizations of nucleolin and fibrillarin (Figs. 1E and S1G), suggesting that H1.X is not required for the nucleolar structure.

We found that H1.X has low chromatin binding activity and exhibits rapid exchange from chromatin compared with H1.2. Recent FRAP analyses have indicated that H1 is dynamically associated with chromatin in vivo and that both the globular and C-terminal domains of H1 are required for its stable association with the nucleosome [17–19]. The C-terminal domain of each H1 subtype is thought to play a role in determining its binding properties, because the features of this domain, such as the number of lysine residues, differ greatly among individual H1 subtypes [20]. For example, 40% of the amino acids in this domain in H1.2 are occupied by two basic amino acids (Lys or Arg), but this proportion is reduced to 33% in H1.X. Furthermore, Thr152 and Ser183, which are important for accurate H1–chromatin interactions [18], are not conserved in H1.X. These features of H1.X are probably responsible for the weaker binding activity between H1.X and chromatin.

H1.X is preferentially distributed to the chromosome periphery, which frequently contains nucleolar components [21], suggesting that H1.X has particular functions at the chromosome periphery. Our previous study on nucleolin, another chromosome peripheral protein, indicated that the integrity of the chromosome periphery is important for chromosome dynamics and cell cycle progression [22]. We did not detect any morphological defects of mitotic chromosomes in H1.X-depleted cells. However, mitotic progression was severely inhibited and defects in chromosome alignment, chromosome segregation, and spindle morphology were observed. Moreover, depletion of H1.X disturbed microtubule attachment to the kinetochores. Taken together, H1.X has different localization patterns and chromatin binding activity compared to the other linker histones throughout the cell cycle and its function is essential for mitotic progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.06.076.

References


