Histone H3 is absent from organelle nucleoids in BY-2 cultured tobacco cells

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Running title: Histone H3 is absent from organelle nucleoids

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; IgG, immunoglobulin G; NGS, normal goat serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; TTBS, Tris-buffered saline containing Tween 20.
Abstract
The core histone proteins (H2A, H2B, H3, and H4) are nuclear-localized proteins that play a central role in the formation of nucleosome structure. They have long been considered to be absent from extra-nuclear, DNA-containing organelles: i.e., plastids and mitochondria. Recently, however, the targeting of core histone H3 to mitochondria, and the presence of nucleosome-like structures in mitochondrial nucleoids, were proposed in cauliflower and tobacco, respectively. Thus, we examined whether histone H3 was present in plant organelles and participated in the organization of nucleoid structure, using highly purified organelles and organelle nucleoids isolated from BY-2 cultured tobacco cells. Immuno-fluorescence microscopic observations and Western blotting analyses demonstrated that histone H3 was absent from organelles and organelle nucleoids, consistent with the historical hypothesis. Thus, the organization of organelle nucleoids, including putative nucleosome-like repetitive structures, should be constructed and maintained without participation of histone H3.

1. Introduction

In eukaryotic cells, nuclear DNA is organized with proteins to form chromatin. The major protein components of the chromatin are core histone proteins (H2A, H2B, H3, and H4). The core histone octamers wind nuclear DNA around them to form nucleosome structures, while linker histones (H1) bind to the DNA between the nucleosomes. During the process of cell division, the nucleosomes become tightly packed, forming highly condensed chromosomes. When active DNA transactions (such as replication, transcription, and repair) are required, histone modifications take place, loosening DNA packaging to enable the DNA transaction apparatus to access the DNA (Ehrenhofer-Murray, 2004). This nucleosome remodeling, triggered by histone modification, is considered to be an essential mechanism for epigenetic regulation of gene expression in eukaryotic cells (Saha et al., 2006).

DNAs present in mitochondria and plastids are also associated with proteins to form compact structures called organelle nuclei or nucleoids (Kuroiwa, 1982, 1991, Sakai et al., 2004). In contrast to nuclear chromatin, however, little is known about the mechanisms or physiological significance of DNA packaging in organelle nucleoids. Historically, histones have long been considered to be absent from mitochondria and plastids, because histone proteins have not been detected in the organelles (and nucleoids) of various organisms, such as the mitochondria of the paramecium (Olszewska and Tait, 1980), slime mold (Kuroiwa et al., 1976), yeast (Caron et al., 1979), and humans (Garrido et al., 2003), as well as in plastids of tobacco (Nemoto et al., 1988, Nakano et al., 1993). Thus, it has been thought that extra-nuclear organelles utilize specific proteins other than histones to organize their DNAs, and many candidate proteins have been identified in mitochondria (e.g., Bogenhagen et al., 2008, Cheng et al., 2007, Chen et al., 2005, Kaufman et al., 2000, Miyakawa et al., 1987, Sasaki et al., 2003, Xu et al., 1996) and plastids (Nemoto et al., 1988, Kobayashi et al., 2002). In plant mitochondria, putative orthologues of nucleoid proteins have been identified in Arabidopsis (Elo et al., 2003), but non-histone protein(s)
responsible for DNA packaging have not been identified clearly.

In contrast to the historical hypothesis described above, Zanin et al. (2010) reported that core histone H3 is targeted not only to the nucleus but also to the mitochondria in cauliflower, based on the results of (i) western blotting analysis, (ii) MS/MS analysis of the proteins from crude mitochondrial fractions, (iii) a computer prediction of the targeting site, and (iv) immuno-histochemistry. They also pointed out the translocation to the mitochondria of histone deacetylase Sir T3 upon cell stress (Scher et al., 2007), suggesting that its substrate H3 might be dually targeted to the mitochondria also.

Recently, we reported that treatment of mitochondrial nucleoids isolated from BY-2 cultured tobacco (Nicotiana tabacum) cells with micrococcal nuclease generated DNA fragments showing a ladder-like pattern upon electrophoretic separation (Takusagawa et al., 2009). Based on the results, we proposed that nucleosome-like structures might be present in the mitochondrial nucleoids from tobacco. Thus, the report by Zanin et al (2010) prompted us to examine the possible involvement of histone H3 in the molecular organization of tobacco mitochondrial nucleoids.

In the present study, we examined whether histone H3 was present in mitochondria and mitochondrial nucleoids in BY-2 cultured tobacco cells, using western blotting and immuno-fluorescence microscopy techniques. Because procedures for the isolation of not only mitochondria and mitochondrial nucleoids, but also nuclei, plastids, and plastid nucleoids with high purity have been established in BY-2 cells (Sakai et al., 1998), we also examined the possible presence of H3 in plastids and plastid nucleoids, which were completely ignored in the report by Zanin et al. (2010). The results strongly suggest that the compact structures of organelle nucleoids in BY-2 cells are constructed in the absence of histone H3.

2. Materials and Methods

2.1 Subcellular localization prediction

Protein sequences were taken from the UniprotKB/Swiss-Prot database. Subcellular localization prediction was conducted with WoLF PSORT (Horton et al., 2007), MitoProt II (Claros and Vincens, 1996), Predotar (Small et al., 2004), and Target P (Emanuelsson et al., 2000).

2.2 Immuno-fluorescence microscopy

Suspensions of protoplasts were mixed with equal volumes of fixative solution (7.4% formaldehyde in 50 mM sodium-phosphate buffer pH 7.0), smeared onto coverslips, and incubated for 20 min at room temperature. After excess fluid was drained off, the sample-bearing cover slips were successively washed in PBS, 75% ethanol, and PBS again for 5 min each. The samples were blocked for 20 min at room temperature with 5% normal goat serum in PBS, incubated with the first antibody (rabbit anti-histone H3 IgG, Sigma H0164, at a dilution of 1:500 with 5% NGS in
PBS) overnight at room temperature, washed for 5 min in PBS, blocked again with 5% NGS for 20 min at room temperature, and incubated with the second antibody (Alexa594-conjugated goat anti-rabbit IgG, Molecular Probes A11037, at a dilution of 1:250 with 5% NGS in PBS) overnight at 4°C. The sample-bearing coverslips were washed in PBS for 5 min at room temperature, rinsed with distilled water, counter-stained with 4′,6-diamidino-2-phenylindole (DAPI), and observed with a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

2.3 Isolation of nuclei, organelles, and organelle nucleoids from BY-2 cells

BY-2 cultured tobacco (Nicotiana tabacum L.) cells were used as the experimental material throughout this study. The procedures for cell culture and isolation of organelles and organelle-nucleoids are described in Sakai et al. (1998). The DNA contents of the isolated organelles and organelle nucleoids were determined with a DNA fluorometer DyNA Quant (GE Healthcare, Buckinghamshire, UK) using calf thymus DNA as a standard, while their protein contents were determined by Lowry’s method using BSA as a standard. Quantitative Southern blotting analyses to assess the purity of the isolated organelle-nuclei were performed according to Sakai et al. (1998).

2.4 Western blotting analysis

Nuclei, organelles, and organelle nucleoids isolated from BY-2 were suspended in SDS-sample buffer (Bio-Rad, Hercules, California, USA). Proteins were separated on 15% SDS-polyacrylamide gels using a Mini-PROTEAN III cell (BioRad, Hercules, California, USA) and visualized by silver-staining (Westmeier 1997). For western blotting analysis, the separated proteins were blotted onto nitrocellulose membranes in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.05% (w/v) SDS] using a MiniTrans-Blot apparatus (BioRad). The membranes were rinsed with TTBS, blocked for 1 hr at room temperature with 3% gelatin in TTBS, and incubated with primary antibody (rabbit anti-histone H3 IgG, H0164, Sigma, at a dilution of 1:10,000 with 1% gelatin in TTBS) for 1 hr at 25°C. After washing in TTBS for 5 min at room temperature, the membranes were incubated with secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase, at a dilution of 1:20,000 with 1% gelatin in TTBS). The bands of histone H3 were detected using an ECL Plus Western blotting detection kit (GE Healthcare) according to the manufacturer's instructions.

3. Results

3.1 Computer-predictions of subcellular localization of histone H3

We first reexamined the computer-predictions of subcellular localization of histone H3 (Tables 1 and 2). As Zanin et al. (2010) reported, MitoProt II and the “normalized feature values” in WoLF
PSORT suggested mitochondrial localization of H3. However, the WoLF PSORT program finally predicted nuclear localization of histone H3 as most probable in most organisms, although localization to mitochondria or plastids was also suggested with low probability in plant histone H3s. The Predotar and Target P programs predicted that non-plant histone H3s did not localize to mitochondria or the ER/secretion pathway. Target P predicted that plant histone H3s were localized to plastids, though with relatively low reliabilities (reliability class 4 or 3 out of 1 to 5, where 1 indicates the strongest prediction), while Predotar did not. The results demonstrated that (i) the prediction was highly dependent on the program used, (ii) mitochondrial localization of histone H3 is not widely supported by various subcellular localization prediction programs, and (iii) the possibility of histone H3 localization to plastids seemed worth examining.

3.2 Immunohistochemical analysis on the subcellular localization of histone H3 in cultured tobacco cells

We examined the possible localization of histone H3 to plastids and/or mitochondria and their nucleoids in BY-2 cultured tobacco cells, because procedures for the isolation of cell-nuclei, organelles, and organelle-nucleoids with high purities have been established in this material (Sakai et al., 1998). First, subcellular localization of histone H3 in BY-2 cultured tobacco cells was examined by immuno-fluorescence microscopy, using the same anti-histone H3 antibody as used in Zanin et al (2010). Strong positive staining was seen only in the nucleus (Fig. 1A a-d), and no signals were observed in the organelles (plastids and mitochondria) or their nucleoids (Fig. 1A e-h).

3.3 Western-blotting analysis on the subcellular localization of histone H3 in cultured tobacco cells

We then examined the possible presence of histone H3 in the isolated organelles and organelle nucleoids by western blotting analysis (Fig. 1B). When each lane was loaded with 25 ng of proteins, histone H3 was clearly detected in the lane of cell-nuclear protein only; The lanes for mitochondria, plastids, and their nucleoids were all negative for histone H3 (Fig. 1B, left). Histone H3 was not detected in the organelles and their nucleoids even if the sample amounts were raised to 300 ng protein per lane (Fig. 1B, right). Judging from the protein/DNA ratios of the cell-nuclei (1.2), mitochondria (12.3), mitochondrial nucleoids (2.4), plastids (5.8), and plastid nucleoids (1.3) used in the analyses, the cell-nucleus equivalent of 25 ng protein should contain about 20 ng of DNA, while the mitochondria, mitochondrial-nucleoids, plastids, and plastid-nucleoids equivalent to 300 ng protein should contain about 20, 130, 50, and 230 ng of DNA, respectively. Thus, whether the comparison was made on a protein basis or DNA basis, the organelles and their nucleoids from BY-2 cells contained little, if any, histone H3 compared to that in the cell-nuclei.
4. Discussion

4.1 Histone H3 is not involved in the organization of mitochondrial nucleoid structure

We have proposed that a small nucleosome-like, repetitive structural unit is present in the mitochondrial nucleoids in BY-2 cultured tobacco cells (Takusagawa et al., 2009). Because Oudet et al. (1978) have demonstrated that a lack of core histones H2A/H2B results in the formation of sub-nucleosomal particles smaller than the genuine ones, we speculated that the presence of histone H3 alone in plant mitochondria (Zanin et al. 2010) might explain the formation of the small nucleosome-like structures we have proposed. In this study, however, we could not detect histone H3 in highly purified mitochondrial nucleoids isolated from BY-2 cells, indicating that the participation of histone H3 in the organization of mitochondrial nucleoid structures is unlikely.

4.2 Histone H3 is absent from mitochondria in BY-2 cultured tobacco cells and most organisms

Our results strongly suggest that histone H3 is absent from mitochondria, plastids, and their nucleoids in BY-2 cells. The absence of histones in extra-nuclear organelles and their nucleoids has also been reported in various organisms (Olszewska and Tait, 1980, Kuroiwa et al., 1976, Caron et al., 1979, Garrido et al., 2003), suggesting absence from extra-nuclear organelles being normal subcellular localization of histone H3 in most organisms, despite the report by Zanin et al. (2010). However, it is still possible that histone H3 is normally present exclusively in the nucleus but that a subpopulation is moved to mitochondria (or plastids) under specific physiological conditions, as reported for a human histone deacetylase SirT3 (Scher et al., 2007).

4.3 Does histone H3 localize to mitochondria in cauliflower?

The argument for the presence of histone H3 in cauliflower mitochondria (Zanin et al., 2010) is based on detection of histone H3 in the crude mitochondrial fraction by western blotting and MS analysis, computer predictions on its subcellular localization, and cytosolic signal deposition in immunohistochemical staining using anti-histone H3 antibody. In addition, no impact of the omission of protease inhibitors during isolation on western blot data supported intra-mitochondrial localization of histone H3. Detection of histone H3 in mitochondrial fraction by western blotting analysis was only successful with antibodies raised against C-terminus of histone H3: The failure of detecting target protein in mitochondrial fraction with antibody raised against N-terminus portion of histone H3 was rationalized by supposing mitochondria-specific N-terminus modification of histone H3 protein.

In spite of the evidences described above, we suspect that histone H3 may also be absent from the mitochondria in cauliflower. First, because cauliflower “mitochondria” were obtained through only three steps of differential centrifugal fractionations, they might be contaminated by nuclear fragments. Although Zanin et al. (2010) checked the contamination by western blotting analysis using antibody raised against a nucleolar protein fibrillarin, a faint band could still be detected in their mitochondrial fraction. Second, computer predictions of the subcellular localization of a
given protein are highly dependent on the program used (Tables 1 and 2) and sometimes erroneous. Third, the resolution of immunohistochemical staining images shown in Zanin et al. (2010) is insufficient to examine subcellular localization of a protein. Fourth, no effect of omission of protease inhibitors and the failure of detecting histone H3 in mitochondrial fraction by N-terminus (but not by C-terminus) specific antibodies allow alternative explanations other than mitochondrial localization of histone H3, such as low proteolytic activities, different binding kinetics between the C- and N-terminus specific antibodies, and (unidentified) N-terminus modification specific for “lighter” chromatin fragments that might selectively contaminate the mitochondrial fraction. In light of the considerations above, we propose that mitochondrial localization of histone H3 in cauliflower awaits further experimental evidence, in which more highly purified specimens, as well as more high-resolution and more quantitative techniques, should be applied.

5. Conclusions

There is little possibility that histone H3 is involved in the organization of mitochondrial nucleoids, including the small nucleosome-like repetitive structures suggested by Takusagawa et al. (2009), in BY-2 cultured tobacco cells. Further analyses to identify protein component(s) responsible for the organization of mitochondrial nucleoids are necessary, which are still ongoing. In addition, more careful and quantitative examination seems necessary to confirm mitochondrial localization of histone H3 in cauliflower.

Acknowledgements

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Table 1  Subcellular localization of histone H3 in various organisms as predicted by WoLF PSORT and MitoProt II.

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For WoLF PSORT: “nucl” and “mito” represent localization feature values suggesting localization to nuclei and mitochondria, respectively, which are normalized to percentiles relative to WoLF PSORT training data. “Location” represents prediction results, in which location sites are abbreviated to four-letter codes (dual localization is denoted by joining the four-letter codes with an underscore character), followed by the numbers of nearest homologues to the query localizing to each site. For MitoProtII: “mito” represents the probability of export to the mitochondria.
Table 2  Subcellular localization of histone H3 in various organisms as predicted by Predotar and Target P.

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For Predotar: “mito”, “chloro”, “ER”, and “elsewhere” represent probabilities of targeting to the mitochondria, chloroplasts (plastids), endoplasmic reticulum, and elsewhere other than those three compartments. For Target P: “cTP”, “mTP”, “SP”, and “Other” represent scores for targeting to chloroplasts (plastids), mitochondria, secretion pathway, and elsewhere other than those three categories, respectively.
Figure legends

Figure 1
Subcellular localization of histone H3 in BY-2 cultured tobacco cells. **A.** Immunofluorescence microscopy showing nuclear localization of histone H3. Protoplasts (a-h) were double-stained with DAPI and anti-histone H3 antibody. (a, e), DIC images. (b, f), DAPI images. Large fluorescent body and small fluorescent spots represent nucleus and organelle-nucleoids, respectively. (c, g), Localization of histone H3. (d, h), Merged images of DAPI and anti-histone H3 antibody staining. Images (a-d) and (e-h) were taken with x40 and x100 objectives, respectively. Scale bars in (a) and (e) represent 10 μm. **B.** Western blot analysis showing nuclear localization of histone H3. Proteins from isolated nuclei (Nuc), mitochondria (Mt), mitochondrial nucleoids (MtN), plastids (Pt), and plastid-nucleoids (PtN) were separated by SDS-PAGE and probed with anti-histone H3 antibody. Top panel, silver-stained image of the gel. Bottom panel, western blot result. The amounts of protein loaded in each lane (together with the approximate amounts of DNA contained in the samples in parenthesis) are indicated on the top of the panels. The sizes of molecular markers are shown to the left of the panels in kDa.