ELECTRON MICROSCOPIC DEMONSTRATION OF CHROMATIN-NUCLEAR MATRIX ASSOCIATION IN THE NUCLEI OF TRITON X-EXTRACTED RAT ANTERIOR PITUITARY CELLS

TAKAO SENDA^{1,3} and MASANORI UMEMOTO²

¹Department of Anatomy I, Nagoya University School of Medicine, Nagoya 466-8550, and ²Department of Otorhinolaryngology, Kinki-Chuo Hospital, Itami 664-0872, Japan

ABSTRACT

We examined intranuclear architecture of Triton X-extracted anterior pituitary cells by quick-freeze deep-etch electron microscopy, scanning electron microscopy and thin section electron microscopy. The nucleoplasm was occupied by a three-dimensional meshwork, which was composed of granular elements, 10–15 nm in diameter, and thin strands, < 10 nm in diameter. The granular elements, most of which must be nucleosomes, were aligned in close proximity to form beads-like chains, 10–20 nm in diameter, or aggregated to form bumpy fibers, 20–30 nm in diameter. On the basis of their dimensions, the beads-like chains and bumpy fibers were presumed to represent chromatin filaments and chromatin fibers, respectively. The < 10 nm thin strands, which belong to the non-chromatin nuclear matrix, interconnected the beads-like chains and bumpy fibers. These results indicate that the nuclear matrix is associated with chromatin loops in the nucleus, and thus support the hypothesis that the nuclear matrix is involved in DNA replication and RNA transcription, processing and transport.

An interphase nucleus consists of a nuclear envelope, nucleoli and chromatin in conventional thin section electron microscopic image. In addition to these, non-chromatin fibrillar components have been reported to form a three-dimensional network in the nucleus. The intranuclear non-chromatin network has been called variously, such as 'ribonucleo-protein network' (36, 40), 'nuclear matrix' (6–10, 21, 29, 30, 35), 'nuclear ghost' (38) and 'nuclear skeleton' (33), but the name 'nuclear matrix' is now widely accepted and used.

The nuclear matrix has been suggested to involve not only in arrangement of the highly-ordered chromatin loops as the skeleton of the nucleus (15, 34), but also in important nuclear functions: DNA replication (5, 6, 8, 16, 25, 37, 41, 42), RNA transcription, processing and transport

(1, 13, 14, 23, 29, 31, 33, 43), T-antigen binding (11) and steroid hormone binding (3). However, these investigations are based mainly on biochemical analyses, and reliable morphological evidence is needed to confirm roles and functions of the nuclear matrix.

Morphological studies on the nuclear matrix have been hampered by several difficulties. A large amount of chromatin fibers with high electron density in thin section electron microscopic image occupies the interior of the nucleus, and consequently obscures nuclear matrix components completely. Therefore, most of the investigators in this field have employed the procedures of chromatin extraction, with which the nuclear matrix can be visualized. However, the extraction of chromatin made it impossible to know relationship between chromatin loops and nuclear matrix components. As a result, chromatin and non-chromatin nuclear matrix have been studied independently by different investigators.

³Correspondence to: T. Senda at the above address. Tel: +81-52-744-2001; Fax: +81-52-744-2012; E-mail: senda@tsuru.med.nagoya-u.ac.jp

In the present study, we have examined non-isolated nuclei in detergent (Triton X)-extracted anterior pituitary cells by quick-freeze deep-etch electron microscopy, scanning electron microscopy and thin section electron microscopy, and demonstrated that nuclear matrix components are associated with the chromatin system.

MATERIALS AND METHODS

The anterior pituitary glands were removed from decapitated male Wistar rats aged 6–10 weeks, and cut into small pieces. They were immersed for 15 min at room temperature in the detergent solution consisting of 100 mM NaCl, 300 mM sucrose, 10 mM Pipes (pH 6.8), 3 mM $MgCl_2$, 1 mM EGTA, 1.2 mM phenylmethylsulfonyl fluoride, 2 mM vanadyl adenosine and 0.5% Triton X-100 (17–19). After wash with the Triton X-uncontained solution, the specimens were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h. Some of the anterior pituitary glands were fixed without the detergent extraction.

Quick-Freeze, Deep-Etch Electron Microscopy

After brief wash with distilled water, the Triton X-extracted and unextracted anterior pituitary tissues were quickly frozen by slamming them against a liquid-helium-cooled pure copper block as described previously (39). The frozen specimens were fractured at $<1 \times 10^{-6}$ Torr at -125°C , deeply-etched at -100°C for 5 min, and shadowed with platinum as thick as 2 nm and with carbon in a freeze-fracture apparatus (JFD-9000, JEOL, Tokyo, Japan). The specimens were immersed in chrome sulfuric acid overnight. The replicas liberated were washed with distilled water, picked up on uncoated copper grids, and examined with a transmission electron microscope (1200EX, JEOL, Tokyo, Japan). Stereo photographs were taken at $\pm 7^\circ$ tilt. Photographs of the quick-freeze deep-etched replicas were printed in negative image.

Scanning Electron Microscopy

The Triton X-extracted and unextracted anterior pituitary tissues were washed with 10% sucrose in distilled water, dehydrated in graded concentrations of ethanol, and frozen in liquid nitrogen. The frozen specimens were cracked with a liquid nitrogen-cooled razor blade, and immersed in isoamyl acetate for 10 min. After critical point drying, the

specimens were coated with platinum as thick as 5 nm in an ioncoater (IB-5, Eiko, Tokyo, Japan), and examined with a scanning electron microscope (S-800, Hitachi, Tokyo, Japan).

Thin Section Electron Microscopy

The Triton X-extracted and unextracted anterior pituitary tissues for thin section electron microscopy were fixed with 2.5% glutaraldehyde and 1% tannic acid in 0.1 M phosphate buffer (pH 7.4) for 2 h. After wash in the buffer, they were post-fixed with 1% OsO_4 in the buffer for 1 h. They were then washed with 10% sucrose in distilled water, dehydrated in graded concentrations of ethanol, and embedded in Epon resin. Ultrathin sections were cut with an ultramicrotome (MT-1, Sorvall, NewTown, CT), stained with uranyl acetate and lead citrate, and examined with the transmission electron microscope.

RESULTS

Quick-Freeze Deep-Etch Electron Microscopic Observation

The nuclei of Triton X-unextracted anterior pituitary cells were filled with a large amount of granular materials, 10–25 nm in diameter, and no other structural component was observed (Fig. 1A). In Triton X-extracted cells, the interior of nuclei was occupied by a three-dimensional meshwork structure (Fig. 1B). Observation at higher magnification revealed that this meshwork structure consisted of essentially two components: granular elements, 10–15 nm in diameter, and thin strands, <10 nm in diameter (Fig. 1C). The granular elements were aligned in close proximity to form beads-like chains, 10–20 nm in diameter (small arrows in Fig. 1C), or aggregated to form bumpy fibers, 20–30 nm in diameter (large arrows in Fig. 1C). The <10 nm thin strands often interconnected between beads-like chains, between bumpy fibers and between beads-like chains and bumpy fibers (arrowheads in Figs. 1C and 2).

Scanning Electron Microscopic Observation

The nuclei of Triton X-unextracted anterior pituitary cells were filled with masses of paste-like substance and a roughly-reticular structure (Fig. 3A). However, no further fine components could be discerned even at a higher magnification (data not shown). In the nucleoplasm of Triton X-extracted

Fig. 1 Quick-freeze deep-etch electron microphotographs showing nuclei of Triton X-unextracted (A) and extracted (B, C) anterior pituitary cells. A: The nucleus of Triton X-unextracted cells is filled with a great amount of granular materials, 10–25 nm in diameter. B, C: The nucleoplasm of Triton X-extracted cells comprises a three-dimensional meshwork structure (B). The meshwork structure is composed of 10–20 nm beads-like chains (*small arrows*), 20–30 nm bumpy fibers (*large arrows*) and < 10 nm thin strands (*arrowheads*) (C). The former two are formed by alignment and aggregate, respectively, of 10–15 nm granular elements. The < 10 nm thin strands interconnect among the beads-like chains and bumpy fibers (*arrowheads*). Bars, 50 nm (A, B), 10 nm (C)

Fig. 2 A stereo pair of quick-freeze deep-etch electron microphotographs showing the nucleoplasm of Triton X-extracted anterior pituitary cells. Note thin strands, < 10 nm in diameter, interconnecting beads-like chains and bumpy fibers (arrowheads). Bar, 50 nm

cells, a three-dimensional meshwork structure was demonstrated also by scanning electron microscopy (Fig. 3B). The meshwork structure consisted of three kinds of components: beads-like chains, 15–30 nm in diameter (small arrows in Fig. 3C), bumpy fibers, 30–40 nm in diameter (large arrows in Fig. 3C), and thin strands, < 15 nm in diameter (arrowheads in Fig. 3C). The beads-like chains and bumpy-fibers were formed by linear arrangement and aggregation, respectively, of the granular elements, 15–25 nm in diameter. The < 15 nm thin strands interconnected between beads-like chains, between bumpy fibers and between beads-like chains and bumpy fibers (arrowheads in Fig. 3C).

Thin Section Electron Microscopic Observation

In the nuclei of unextracted cells, though nucleoli, heterochromatin and euchromatin were identified,

further fine substructures could not be revealed (Fig. 4A). The nucleoplasm of Triton X-extracted cells was occupied by a meshwork structure (Fig. 4B). At a higher magnification, the meshwork structure consisted of beads-like chains, 10–20 nm in diameter (small arrows in Fig. 4C), bumpy fibers, 20–30 nm in diameter (large arrows in Fig. 4C), and thin strands, < 10 nm in diameter (arrowheads in Fig. 4C). The beads-like chain and bumpy fiber were formed by alignment and aggregate, respectively, of highly electron-dense granular elements, 10–15 nm in diameter. The < 10 nm thin strands interconnected between beads-like chains, between bumpy-fibers and between beads-like chains and bumpy fibers (arrowheads in Fig. 4C).

DISCUSSION

In the present study, we tried to reveal relationship between the chromatin system and non-chromatin nuclear matrix in nucleus. Our strategy employed

Fig. 3 Scanning electron microphotographs showing nuclei of Triton X-unextracted (A) and extracted (B, C) anterior pituitary cells. A: The nucleoplasm of Triton X-unextracted cells is filled with masses of paste-like substance and a roughly-reticular structure. B, C: The nucleoplasm of Triton X-extracted cells comprises a three-dimensional meshwork structure (B). The meshwork structure is composed of 15–30 nm beads-like chains (*small arrows*), 30–40 nm bumpy fibers (*large arrows*) and < 15 nm thin strands (*arrowheads*) (C). The former two are formed by alignment and aggregate, respectively, of 15–25 nm granular elements. The < 15 nm thin strands interconnect the beads-like chains and bumpy fibers (*arrowheads*). Bars, 200 nm (A, B), 20 nm (C)

Fig. 4 Thin section electron microphotographs showing nuclei of Triton X-unextracted (A) and extracted (B, C) anterior pituitary cells. A: In the nuclei of Triton-unextracted cells, detailed substructure except for nucleoli, heterochromatin and euchromatin cannot be revealed. B, C: The nucleoplasm of Triton X-extracted cells comprises a meshwork structure (B). The meshwork structure is composed of 10–20 nm beads-like chains (*small arrows*), 20–30 nm bumpy fibers (*large arrows*) and < 10 nm thin strands (*arrowheads*) (C). The former two are formed by alignment and aggregate, respectively, of 10–15 nm granular elements. The < 10 nm thin strands interconnect the beads-like chains and bumpy fibers (*arrowheads*). Bars, 100 nm (A, B), 10 nm (C)

here is as follows. First of all, any treatments resulting in removal of chromatin components were avoided. Therefore, we used only Triton X-100, a non-ionic detergent, in order to permeabilize the plasma membrane and nuclear membranes resulting in extraction of only phospholipids and soluble nuclear proteins unbound to the chromatin system from nucleus (17, 19). The removal of soluble nuclear proteins with Triton X was also expected to bring both the chromatin components and nuclear matrix into high relief in electron microscopic image.

Secondly, we utilized quick-freeze deep-etch electron microscopy and scanning electron microscopy. Quick-freeze deep-etch electron microscopy, which provides three-dimensional architecture of subcellular components at high resolution, is believed to be one of the most useful technique for observation of filamentous components. Though numerous studies dealing with cytoskeleton by quick-freeze deep-etch electron microscopy have been reported, intranuclear architecture has not been investigated using this technique. Scanning electron microscopy is also a good technique for visualization of filamentous components. Indeed, several investigators have examined intranuclear architecture by scanning electron microscopy (2, 26, 44). However, they focused on the construction of chromosomes and/or chromatin, and did not mention the non-chromatin components.

Thirdly, we observed nuclei in cells and tissues, and avoided isolation of nuclei. The isolation of nuclei is an initial step in the standard procedure for nuclear matrix preparation (9). However, this may cause artificial changes in the nuclear matrix structure and relationship of the nuclear matrix with the chromatin system. In fact, firm association of cytoplasmic filaments with the nuclear matrix was found by analyses using whole cells (12, 19). As shown in the previous (17, 19) and our present studies, 0.5% Triton X-extraction was sufficiently effective on non-isolated nuclei in the cells.

It has been known that there exist two kinds of fibrous system in the nucleus; chromatin filaments or fibers and non-chromatin nuclear matrix. The chromatin filaments consist of DNA and histone proteins. It is suggested that DNA winds around the histone proteins to form nucleosomes, about 10 nm in diameter, and that many nucleosomes are aligned along the DNA to form a chromatin filament, which then coils up and aggregates to form a chromatin fiber (27, 28). Most likely, the granular elements in the present study, the real diameter of

which without the thickness of platinum coat is about 10 nm, correspond to the nucleosomes, and thus the beads-like chains and bumpy fibers represent chromatin filaments and chromatin fibers, respectively.

The thin strands observed in this study are unlikely to be chromatin filaments for the following reasons. In the present study, the thickness of platinum coat was controlled at 2 nm and 5 nm in quick-freeze deep-etch technique and scanning electron microscopy, respectively. Consequently, the real diameter of the thin strands without the platinum coat is < 10 nm, which is obviously less than that of the chromatin filaments. On the other hand, filamentous components consisting of the nuclear matrix have been reported to be < 10 nm in diameter (9, 17, 36). Furthermore, the thin strands appeared thread-like with smooth surface, while the chromatin filaments formed by nucleosomes were beads-like and bumpy. Therefore, we conclude that the thin strands are included in the non-chromatin nuclear matrix.

Because both of the intranuclear fibrous systems have previously been investigated separately, morphological relationship between them remains unknown. In the present study, we found thin strands which interconnect the beads-like chains and bumpy fibers; that is to say, non-chromatin nuclear matrix components which interconnect chromatin filaments and fibers. The nuclear matrix may act as a connector within the three-dimensional network of chromatin filaments and fibers, and contribute to organization of the chromatin loops in the nucleus. Association of chromatin loops with the nuclear matrix supports the hypothesis that the nuclear matrix carries the DNA replication sites (6, 8), and has a role for RNA transcription, processing and transport (1, 13, 14, 23, 29, 31, 33, 43). Though the nuclear matrix is suggested to comprise more than 200 proteins (18), only a few proteins, such as lamins (20, 24, 32) and matrins (4, 22) were identified. In addition to the biochemical identification of unknown nuclear matrix proteins, precise localization of the nuclear matrix proteins and visualization of the structure composed of these proteins will provide important information to understand roles of the nuclear matrix.

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