Ultrastructural Aspects of Quick-Freezing Deep-Etching Replica Images of the Cytoskeletal System in Anterior Pituitary Secretory Cells of Rats and Mice*

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Summary. In order to study the three-dimensional architecture of the cytoskeletal system and its functional properties in the secretory cell, the anterior pituitaries of rats and mice were examined by the quick-freezing deep-etching method.

The cytoplasm of the anterior pituitary cell is occupied by networks of several kinds of fine filaments. For convenience, the filaments which terminate at cytoorganelles, secretory granules, and plasma membrane were classified into three types.

The filaments running away from the complicated networks of fine filaments terminate on the outer surface of the membrane of the cytoorganelles, secretory granules, and small vesicles and on the inner surface of the plasma membrane. These filaments, 4-10 nm in diameter, were termed associating filaments by us.

There are direct connections by filaments between adjacent cytoorganelles, or between the cytoorganelle and secretory granule, or between the secretory granule and plasma membrane. These filaments are also 4-10 nm in diameter, and were termed connecting filaments by us.

Short filaments, 3-8 nm in diameter, link directly at right angles the opposite membranes crossing over the cisterna of the nuclear envelope, of the rough endoplasmic reticulum, of the Golgi apparatus, and the intramitochondrial space and also the intercellular space. We named these linking filaments.

These findings strongly suggest that these filaments are essential elements for supporting, maintaining and organizing the shape and location of all the cytoorganelles in the cell, and that they vary according to cellular function.

The limiting membrane of the secretory granule is bound to the associating filaments and connecting filaments, running radially and at times connecting to the microtubules or plasma membrane. These filaments and microtubules may play a role in the organization and transport of secretory granules toward the plasma membrane.

Recently, the cytoskeletal system, consisting of microtubules, intermediate filaments, and microfilaments, has come to be regarded as one of the most important components of the cytoplasm, because of its involvement in various kinds of cell functions such as support, movement, proliferation, secretion, pinocytosis, phagocytosis, transport, morphogenesis, etc.

Though the role of this structure in secretory activity has been discussed by several authors (e.g., LACY et al., 1968; DUSTIN, 1978; SASAKI et al., 1981), most papers have dealt with the structure and function of the cytoskeleton in the secretory cell.

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using thin section images. Fine morphological work showing the three-dimensional conformation of the cytoskeleton and its relationship to the secretory activity has been wanting.

The quick-freezing deep-etching technique, which was developed by HEUSER and SALPETER (1979), is now believed to be one of the most useful methods for this task. Though numerous papers concerning the deep-etching images of quick-frozen tissues have been published (the neuromuscular junction: HEUSER and SALPETER, 1979; HIROKAWA and HEUSER, 1982a, brush border of the small intestine: HIROKAWA and HEUSER, 1981; HIROKAWA et al., 1982, 1983; KANASEKI, 1983, axoplasm: HIROKAWA, 1982a; SCHNAPP and REESE, 1982; TSUKITA et al., 1982; KANASEKI 1983; HIROKAWA et al., 1984, hair cells of the inner ear: HIROKAWA and TILNEY, 1982, rod disks: USUKURA and YAMADA, 1981; ROOF and HEUSER, 1982; ROOF et al., 1982, olfactory and respiratory epithelial cells of the nose: MENCO, 1984, junctional complexes: HIROKAWA and HEUSER, 1982b; HIROKAWA, 1982b, pigment cells: IP et al., 1984, cilia and flagella: TSUKITA et al., 1983; ARIMA et al., 1984; GOODENOUGH and HEUSER, 1985, dendritic spines: LANDIS and REESE, 1983, microvilli: CHANDLER and HEUSER, 1981; SHIBATA et al., 1983, endothelial fenestellae: BEARER and ORCI, 1985, myocardial cells: FRANK and BEYDLER, 1985, epithelial cells of the choroid plexus: MELLER, 1985, stomach surface mucous cells, KANASEKI, 1983, secretory cells of pancreas: KANASEKI, 1983, liver cells: KANASEKI, 1983, erythrocyte: KANASEKI, 1983 and so on), secretory cells have yet to be sufficiently investigated using this technique, notwithstanding a few reports dealing with the stomach surface mucous cells and pancreatic exocrine cells by KANASEKI (1983).

Anterior pituitary cells are known to be typical protein secretory endocrine cells containing numerous secretory granules in the cytoplasm, and they seem to be suitable for detecting potential relationships between secretory activity and the cytoskeleton by the quick-freezing deep-etching technique.

MATERIALS AND METHODS

Ten male Wistar rats aged 6-10 weeks and 10 male ddY mice aged 8 weeks were used for the study. The anterior pituitaries were resected, cut in half, and then put on the specimen holder as their sections were exposed. The tissues with which the quick-freezing apparatus "Slammer" (Polaron) was fitted were frozen quickly by slamming against the surface of a pure copper block cooled with liquid helium. The frozen specimens were stored in liquid nitrogen. Those specimens which were transferred into a JEOL JFD-7000 freeze-fracture apparatus were fractured with a cooled blade within 20 μm from the slammed surface at −120°C, deeply etched for 10 min at −90°C at 1 × 10⁻⁶ Torr, rotary-shadowed with platinum/carbon at an angle of 30° and shadowed with carbon at an angle of 90°. The shadowed specimens were removed from the apparatus and immersed in 30% chromic acid. After the tissues were completely dissolved, the replicas were rinsed carefully with distilled water, picked up on 300-mesh copper grids, and examined with a Hitachi H-500 type electron microscope. All photographs were printed in reverse image.
OBSERVATIONS

Orientation
As it is difficult to remove granular materials of 3-20 nm diameter, distributed throughout the cytoplasm and around the cytoskeletons without damage to the cytoorganelles using saponin in most endocrine cells as compared to axons and intestinal epithelial cells, the present observations were done using the quick-freezing deep-etching material from the fresh materials.

By the quick-freezing deep-etching method, the plasma membrane, nucleus, mitochondria, rough endoplasmic reticulum, Golgi apparatus, secretory granules, microtubules, and complicated networks of fine filaments are observed (Fig. 1-7).

Since each filament is decorated with many granular materials, it is often difficult to clarify its true diameter and structure. Therefore the diameter of the microtubules and filaments described in this paper were measured using the bare portion that appeared devoid of granular materials.

Occasionally, microtubules 25-27 nm in diameter can be seen among the networks of filaments in the replicas (Fig. 6). These structures are not abundant, and their entire course and direction are hard to clarify.

For convenience, we classified the filaments into three groups—associating filaments, connecting filaments, and linking filaments—on the basis of their location (Fig. 8).

The cytoplasmic filaments which terminate on the outer surface of the membranes

![Image](https://via.placeholder.com/150)

**Fig. 1.** Quick-freezing deep-etching image of the nucleus (N), nuclear envelope (E), and rough endoplasmic reticulum (R) of a rat anterior pituitary cell. Note connecting filaments (C) between the outer membrane of the nuclear envelope and the membrane of the rough endoplasmic reticulum, and linking filaments in the cisternae of the nuclear envelope (arrow) and those of the rough endoplasmic reticulum (arrow). The interior of the nucleus is occupied with anastomosing filaments. Each filament is decorated with many granular materials. ×114,000
of the cytoorganelles, secretory granules and plasma membrane, were classified as associating and connecting filaments. The filaments running from the complicated networks of fine filaments often terminate at the outer surface of the membrane of the cytoorganelles, secretory granules, and small vesicles, and also at the inner surface of the plasma membrane. These filaments, 4-10 nm in diameter, were termed by us associating filaments (Fig. 3, 4, 7, 8). There are direct connections by filaments between adjacent structures, such as between the plasma membrane and the membrane of the secretory granule, between the membrane of the mitochondria and that of rough endoplasmic reticulum, between the microtubules and the membrane of the secretory granules, between the membranes of two sacs of rough endoplasmic reticulum, and between the membranes of two sacs of the Golgi apparatus. These filaments, 4-10 nm in diameter, were named by us connecting filaments (Fig. 1, 2, 4-6, 8).

In the cisternae of the nuclear envelope, the rough endoplasmic reticulum, and the Golgi apparatus, in the mitochondrial space between the outer and inner membranes, and in the intercellular space between the plasma membranes of two cells, the opposite membranes are linked by short thin filaments 3-8 nm in diameter crossing through the cisterna of space. We named them linking filaments (Fig. 1-3, 5, 8).

The filaments in each organelle and the relationship between the cytoplasmic filaments and cytoorganelles or cytoplasmic structures are described as follows.
Fig. 3. Quick-freezing deep-etching image of a mitochondrion (M) of a rat anterior pituitary cell. Outer and inner membranes of the mitochondrion are linked by linking filaments (arrow) crossing the intermembranous space. The membranes of cristae are also connected by linking filaments (arrow head) crossing the intercristal space. Many associating filaments terminate at the outer membrane of the mitochondrion. R rough endoplasmic reticulum. ×120,000

Fig. 4. Associating filaments (arrow) terminating at the outer surface of the limiting membrane of secretory granules (G), running almost regularly in a radial array. Connecting filaments between a mitochondrion (M), and rough endoplasmic reticulum (R), and between mitochondrion and secretory granules. I intercellular space. ×83,000
Nucleus (Fig. 1)

The cross fractures provide visual access to the interior of the nucleus and the cisterna of the nuclear envelope.

The interior of the nucleus is occupied with branching and anastomosing filaments decorated with many granular materials. The filaments are more densely distributed in the peripheral region near the nuclear membrane than in the central region of the nucleus. There are no differences in diameter between the intranuclear filaments and the cytoplasmic ones.

In the cisterna of the nuclear envelope, short, straight, and unbranched linking filaments connecting the inner nuclear membrane with the outer one are seen. They are arranged at relatively regular intervals, and run at almost right angles to both nuclear membranes. Compared with those of the cytoplasmic or intranuclear filaments, attached granular materials are fewer.

Many associating filaments terminate at the outside of the outer nuclear membrane. Moreover, cytoorganelles lying near the nucleus are connected with the outer nuclear membrane by the connecting filaments.

Rough endoplasmic reticulum (Fig. 1, 2, 4)

Ribosomes, 15-20 nm in diameter, adhere to the outer surface of the membrane of rough endoplasmic reticulum. In the replica image, they are not easy to distinguish from the granular materials of the associating or connecting filaments of this organelle.

The associating filaments terminate at the outer surface of the membrane of the rough endoplasmic reticulum. Flattened sacs of the rough endoplasmic reticulum are interconnected with one another by many connecting filaments.

The outer surface of the membrane of the rough endoplasmic reticulum and that of the neighboring organelles such as mitochondria and small vesicles (probably transporting vesicles which carry the exportable protein from rough endoplasmic reticulum to the Golgi apparatus) are also connected to each other by the connecting filaments.

The cisternae of the rough endoplasmic reticulum are generally attenuated and narrow in replica imagery, and there are short, straight and unbranched filaments interlinking the membrane of this organelle with that of the opposite side while crossing through the cisterna. These linking filaments are arranged at relatively regular intervals and run at almost right angles to both membranes.

Golgi apparatus (Fig. 2)

The Golgi apparatus consists of stacks and vesicles. The deep-etching method reveals the Golgi sacs linked together by the connecting filaments. Transverse fractures of the Golgi cisternae are narrow and the linking filaments can be recognized in them. Some small vesicles localized between the cis side of the Golgi apparatus and the rough endoplasmic reticulum are considered to be transporting vesicles. They are associated with the membranes of both organelles by the connecting filaments.

Mitochondria (Fig. 3, 4)

Mitochondria are scattered throughout the cytoplasm. The outer and inner mem-
branes can be clearly identified and both membranes are connected by *linking filaments*. The cristae are interconnected by *linking filaments*. Many *associating filaments* terminate at the outer membrane of the mitochondrion, and the *connecting filaments* link the outer membrane of this organelle with the membrane of the adjacent structures, such as the secretory granules, rough endoplasmic reticulum, Golgi apparatus, mitochondria and so on.

Fig. 5. A *connecting filament* joining the limiting membrane of a secretory granule with a plasma membrane is seen (arrow). *Linking filaments* (arrow head) linking opposite membranes crossing through the intercellular space (I) of adjacent anterior pituitary cells. ×187,000

Fig. 6. A secretory granule (G) connected with a microtubule (T) by *connecting filaments* (arrow). ×91,000

Fig. 7. Many *associating filaments* (white arrows) terminating at the true inner surface of the plasma membrane. Notice the boundary (black arrows) between the true inner surface and the E face of the plasma membrane. ×77,000
Secretory granules (Fig. 2, 4-6)

The relationship between secretory granules and the associating or connecting filaments is characteristic. The outer (cytoplasmic) surface and the E face of the limiting membrane of the secretory granule is exposed by the fracturing and etching. It is clear that the associating filaments terminate on the outer surface of the limiting membrane. They run almost regularly in a radial array. The associating filaments of the secretory granule are relatively free from granular materials as compared with those of other regions. The connecting filaments, which connect the limiting membrane of the secretory granule with that of adjacent secretory granules, the plasma membrane, the membranes of other organelles, or with microtubules, can also be seen.

Plasma membrane (Fig. 5, 7)

We were able to view the P face, E face, true inner (cytoplasmic) surface, and true outer (interstitial) surface of the plasma membrane in deep-etched replicas. Many filaments in the cytoplasm are associated with the true inner surface of the plasma membrane. The cytoorganelles are connected with the plasma membrane by connecting filaments. Straight and unbranched linking filaments connect the plasma membrane with that of the adjacent cell, crossing the intercellular space and running at right angles to them.

Generally, all the cytoplasmic-formed structures, such as cytoorganelles and secretory granules, are not located independently, but interconnected by the associating, or connecting filaments, or a network of them.

Fig. 8. Schematic drawing of the cytoskeletal system of the anterior pituitary cell of rats and mice. 1: Associating filaments, 2: connecting filaments, 3: linking filaments, N networks of filaments in the cytoplasmic matrix.
DISCUSSION

The rapid-freezing deep-etching method reveals a complicated network of filaments which associates with all the formed structures in the cell. As it is impossible to determine the chemical properties of each of the filaments observed in this study, we classified them, for convenience, into three groups on the basis of their location and association with other structures. The cisternae of the nuclear envelope, of the rough endoplasmic reticulum, and of the Golgi apparatus, the intramitochondrial space between the inner and outer membranes and between the cristae, and the intercellular space were found to be supported by short linking filaments. The flattened sacs of the rough endoplasmic reticulum were considered to be connected and packed by the connecting filaments, and occupy certain regions of the cytoplasm. The Golgi lamellae are also arranged in the same manner. These findings strongly suggest that the shape and localization of these formed structures are well maintained and organized by these filaments. If these filaments should be absent, we speculate that the formed structures will then disperse into the cytoplasm and their characteristic structures be difficult to maintain.

The function of the cytoskeleton for the secretion and intracellular transport of the cytoplasmic-formed elements is of great interest. The vesicles located in the region between the rough endoplasmic reticulum and Golgi apparatus are associated with both organelles by the connecting filaments. This fact may indicate the possibility that the connecting filaments are related to the transport of the vesicles from the rough endoplasmic reticulum to the Golgi apparatus. Possible functions of microtubules for intracellular transport of the secretory granule have been proposed (LACY and MALAISSE, 1973; DUSTIN, 1978; SASAKI et al., 1981). The present study demonstrates the occurrence of connecting filaments between the secretory granule and microtubule. We speculate that the interaction among the associating filaments, connecting filaments, microtubules, and surrounding cytoplasmic filaments might play some role in the transport of secretory granules toward the plasma membrane. The secretory granules located just beneath the plasma membrane are connected with the plasma membrane by the connecting filaments, which may perform an important function when exocytosis occurs. ORCI et al. (1972) proposed a "cell web", or the particular layer just beneath the plasma membrane in the pancreatic β cell, that is composed of fine filaments, and speculated on its possible relation with exocytosis. However, in the present study, the anterior pituitary cells did not show such a dense network of fine filaments.

We wish to especially emphasize the finding that the cytoorganelles and secretory granules in a cell are obviously related with the associating filaments and entirely connected with the network of filaments, while the adjacent membranes in each organelle such as in the rough endoplasmic reticulum, Golgi apparatus, mitochondria, or nuclear envelope, are connected with the short linking filaments crossing the cisterna or intramitochondrial space. This finding suggests that these filaments have an important function in supporting, organizing, and controlling these organelles and formed structures as parts of the whole cell.

Problems remain concerning the nature of the filaments observed in the present study. A possibility that some filaments such as the intercellular or intracisternal filaments may be condensation artifacts of cytoplasmic protein or salts can not be ruled out. The polyethylene glycol method revealed that 8-10% albumin solutions
exhibited an image of a lattice of filamentous structures (Kondo, 1984). Miller et al. (1983) reported that sodium chloride and sucrose subjected to quick-freezing and freeze-drying methods produced filament-like structures. Both filament-like structures resemble the cytoplasmic filaments found in the present study. Further studies are needed in regard to this problem.

The chemical nature of cytoskeletal filaments has been studied immunocytochemically by several investigators using the quick-freezing deep-etching method. The cytoskeleton of the terminal web of the intestinal epithelium is known to consist of actin, myosin, intermediate, and FST (fodrin-spectrin-TW260/240) filaments (Hirokawa et al., 1982, 1983). Three kinds of polypeptides (195, 145, and 73Kd) have been found to be chemical components of neurofilaments and their cross-bridges in the axon of the rabbit spinal cord (Hirokawa et al., 1984). Actin filaments have also been found morphologically or by S1-decoration in the pre- and postsynaptic nerve terminals at the neuromuscular junction (Hirokawa and Heuser, 1982a), in hair cells (Hirokawa and Tilney, 1982), in liver cells (Hirokawa and Heuser, 1982a), in cerebellar dendritic spines (Landis and Reese, 1983), in cultured fibroblasts (Heuser and Kirchner, 1980), and in muscle cells (Heuser, 1983). As it is impossible to determine the chemical properties of each of the filaments observed in this study, there might be overlaps and confusion in chemical properties among these different groups of filaments. In order to clarify the nature of each filament, further studies are needed. Though this problem has yet to be solved, the statements described above seem sufficiently relevant to discuss the cytoskeleton of the secretory cell in the fresh condition.

Some of the granular materials decorating the filaments may be soluble proteins (Hirokawa and Heuser, 1981) or free ribosomes in the cytoplasm.

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