Studies on the Intracellular Protein by Immuno-Electron Microscopy

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Summary

Euglena cells were grown synchronously under photoautotrophic culture conditions on a 14 h light-10 h dark alternations. Changes in morphology of the pyrenoid and those in distribution of RuBisCo in chloroplasts were followed by immunoelectron microscopy during the growth and division phases of *Euglena* cells. The immunoreactive protein were densely localized in the pyrenoid, and thinly distributed in the stroma during the growth phase. During the division phase, the pyrenoid could not be detected and the gold particles were dispersed throughout the stroma. From a comparison of photosynthetic CO_2-fixation with the total carboxylase activity of RuBisCo extracted from *Euglena* cells in the growth phase, it is suggested that the carboxylase in the pyrenoid functions in CO_2-fixation in photosynthesis. Cells of *Euglena* contain a LHC II. The precursors to LHC II are

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large polyproteins containing multiple copies of LHC II, and photocontrol of their formation is largely translational. Under conditions favoring LHC II accumulation in the thylakoids, a reaction with anti-LHC II antibody can be observed in the Golgi apparatus by immunogold electron microscopy. The timing of the immunoreaction in the Golgi apparatus in synchronous cells and in cells undergoing normal light-induced chloroplast development suggests that the nascent LHC II passes through the Golgi apparatus on the way to the thylakoids.

Key Words :

Euglena, Immuno-electron microscopy, LHC II, RuBisCo, Golgi apparatus.

Introduction

Recently, as structures of photosynthesis functional units, represented by chloroplast photochemical proteins, are being elucidated at the molecular level, dynamic states, transport, and complex formation mechanisms of these molecules have drawn attention. Mitochondrial and chloroplast DNAs were discovered about 35 years ago, and the genetic information and the existence of their expression systems have been revealed. With the determination of all the structure of chloroplast DNA, it has been discovered that chloroplasts and mitochondria are formed by interaction among genes that exist in the cell nucleus. It is known that, after protein molecules controlled by the cell nucleus are synthesized in the cytoplasm, they are transported to organelles to undergo processing within them, and reach the final location to be incorporated[1]. By using immuno-electron microscopy, the dynamics of these protein molecules can be linked directly with cell structure for analysis. More specifically, protein-A gold immuno-electron microscopy using colloidal gold particles is superior to conventional autoradiography or ferritin antibody method in both resolution and contrast, and location of intracellular

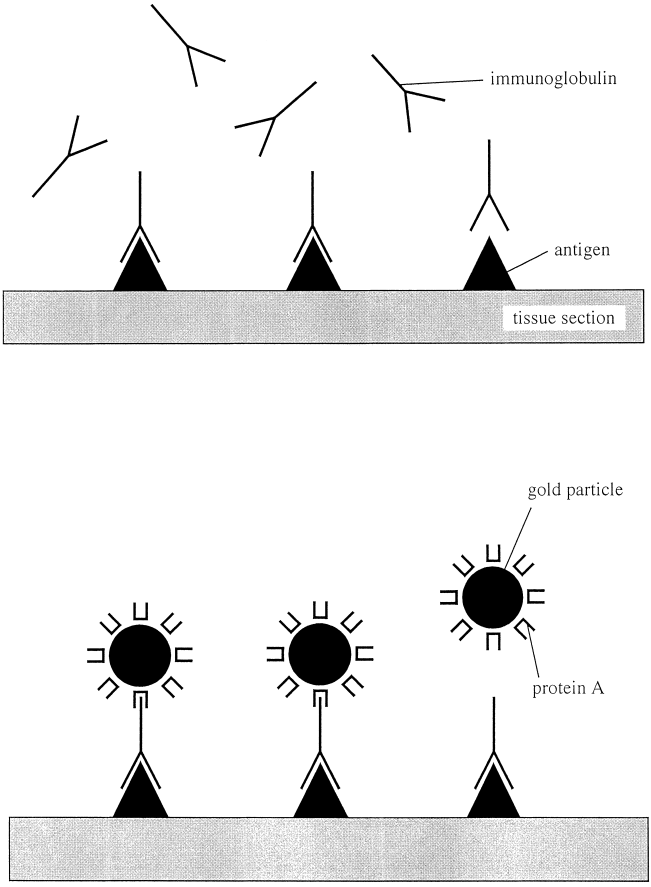

Fig. 1

Diagram illustrating the principles of the protein A-gold approach. The labeling is carried out in two steps.

antigens can be comprehended with accuracy, since their location is replaced by high electron-density gold particles[2, 3]. However, much remains to be clarified, since tracing the location of antigens (active molecules in the living body) by

electron microscopy poses a conflicting restriction of preserving cellular ultrastructure and retaining antigen substances within the cell at the same time. Today, cryo-ultramicrotomy and rapid freeze-replacement fixation methods, which prevent the inactivation of antigen substances and fix them closely under in vivo conditions, are being introduced, but these methods are too complex and have a disadvantage of poor image reproducibility, and therefore, are not widely used.

This article briefly introduces research on *Euglena gracilis* photosynthetic proteins, conducted in our laboratory using protein-A gold method (Fig. 1), one of the common heavy metal-labeled antibody methods in immuno-electron microscopy applied to plant cells[4-6].

Immuno-electron microscopy

First, an enzyme antibody method that labels antibodies with enzymes and a heavy metal-labeled antibody method that labels antibodies with heavy metal are available, and the latter includes a ferritin antibody method, colloidal gold method, and others[2, 3]. Also, an antigen-antibody reaction method can be divided into a pre-embedding staining method in which reaction is performed before resin-embedding of samples, and a post-embedding staining method in which reaction is performed on sections after resin-embedding and preparation of ultrathin sections. Since immune responses are performed before sample fixation in the pre-embedding staining method, antigens are better preserved compared with the post-embedding method, and the sensitivity is high, and in addition, the microstructure is better preserved. However, higher plants and algae have a disadvantage of poor penetration of antibodies directly into the cells due to their special structures such as cell wall and vacuole. As a result, there is a phenomenon that antigen detection sensitivity is high in a small part of the cell surface layer, however, low or zero

within it or high-density secretory granules. Therefore, the post-embedding staining method is commonly used for plant cells. The important aspect in sample preparation for immuno-electron microscopy include retaining antigenecity, preserving cellular microstructure, and conditions such as high specificity and titer of the antibody used, and lacking even one of them makes it impossible to obtain clear images. Therefore, it is essential for researchers to select a fixation method and an embedding resin, appropriate to their samples, and adjust antibody titers. This article describes the procedures of the post-embedding staining method in protein-A gold method[7, 8].

Methods

Ⅰ. Sample Preparation :

1. Add 0.9 mL 50% glutaraldehyde to 45 mL *Euglena* cells in culture for a final concentration of approximately 1% glutaraldehyde and incubate at 4℃ for 60 min. Recover the cells by centrifugation for 2 min in a table top centrifuge.
2. Resuspend the cell pellet in 10 mL 0.1 M phosphate buffer pH 7.2, incubate 5 min at room temperature on a specimen rotator and recover the cells by centrifugation for 2 min in a table top centrifuge. Repeat two times.
3. Resuspend the cell pellet in 1 mL 0.1 M potassium phosphate buffer pH 7.2 and transfer to a microfuge tube.
4. Embed the cell pellet in 2% (w/v) agarose by resuspending the cell pellet in 60℃, 2% agarose and immediately centrifuge for 30 sec to pellet the cells. Remove the tube and place on ice to solidify the agarose.
5. Remove the agarose plug from the microfuge tube with a needle and cut off the region containing the cells. Cut the agarose into small cubes and transfer to a 15 mL conical centrifuge tube.

6. Dehydrate the samples by incubating on a specimen rotator for 20 min in 10 mL 50% ethanol and recover the sample by gentle centrifugation. Repeat once.
7. Dehydrate the samples by incubating on a specimen rotator for 20 min in 10 mL 70% ethanol and recover the sample by gentle centrifugation. Repeat once.
8. Dehydrate the samples by incubating on a specimen rotator for 20 min in 10 mL 90% ethanol and recover the sample by gentle centrifugation. Repeat once.
9. Resuspend the sample in 10 mL acetone, incubate on a specimen rotator for 20 min and recover the sample by gentle centrifugation. Repeat three times.

Ⅱ. Embedding Samples :

1. Resuspend samples in 3 mL of a 1 : 2 resin : acetone mixture, place uncapped on a specimen rotator in a hood and incubate 4 h to overnight. Recover the sample by gentle centrifugation and remove the resin using plastic pipettes.
2. Resuspend the samples in 3 mL of a 2 : 1 resin : acetone mixture, place uncapped on a specimen rotator in the hood and incubate 4 h. Recover the sample by gentle centrifugation and remove the resin using plastic pipettes.
3. Resuspend the sample in 3 mL 100% resin and incubate in a vacuum desiccator for 1-2 h. Recover the sample by gentle centrifugation and remove the resin using plastic pipettes.
4. Resuspend the sample in a small volume of resin. Fill a gelatin capsule about half full and overlayer with the cell sample. Place the capsule in a centrifuge tube positioning it upright in the tube using tissue paper and centrifuge at full speed in a clinical centrifuge for 10 min to pellet the cells to the bottom of the capsule.
5. Remove the capsule from the centrifuge tube, top off the capsule with resin, insert a sample identification label into the resin at the top of the capsule and polymerize by incubation in a 60℃ oven for 24 h.

6. Allow the polymerized block to cool for 24 h. Remove the block from the gelatin capsule by placing in water at 37℃ until the capsule dissolves.

Ⅲ. Preparation of ultrathin sections :

1. Mount the block in a block trimmer and shape the end into a four sided pyramid with walls at a 45° angle and a 0.5-0.75 mm square top surface.
2. Mount the trimmed block on a microtome making sure that the block face is parallel to the knife edge. Fill the diamond knife trough with distilled water so it is level with the cutting edge.
3. Cut a ribbon of silver ultra-thin sections approximately 80-90 nm thick.
4. Use an eye-brow tool to separate the ribbon into 5 or 6 sections and align them in the trough.
5. Place a grid held with a pair of tweezers under the sections and raise it positioning the sections in the middle of the grid.
6. Blot the jaws of the tweezers and the bottom of the grid with Whatman # 1 filter paper to absorb all of the liquid. Place the grid sample side up on a dry piece of filter paper in a Petri dish and allow to dry overnight.

Ⅳ. Immunogold labeling of sections :

1. Ultrathin sections on grids are floated section side down on a 300 μL drop of freshly prepared 0.3% hydrogen peroxide solution for 10 min.
2. Wash ultrathin sections on grids four times with PBS by floating grids section side down on a 300 μL drop of PBS for 30 min.
3. Block ultrathin sections on grids by floating grids section side down on a 300 μL drop of PBS-BSA for 30 min.
4. Incubate ultrathin sections on grids with primary antibody by floating grids section side down on a 300 μL drop of primary antibody diluted in PBS-BSA and

incubating at 37℃ for 20 min.

5. Wash ultrathin sections on grids twice with PBS-Tween by floating grids section side down on a 300 μL drop of PBS-Tween for 30 min.

6. Incubate ultrathin sections on grids with protein-A gold by floating grids section side down on a 300 μL drop of protein-A gold diluted 1 : 10 or 1 : 20 in PBS and incubating for 20 min at room temperature.

7. Wash ultrathin sections on grids twice with PBS-Tween by floating grids section side down on a 300 μL drop of PBS-Tween for 10 min.

8. Wash ultrathin sections on grids twice with deionized water by floating grids section side down on a 300 μL drop of deionized water for 10 min.

9. Stain ultrathin sections on grids with uranyl acetate by floating grids section side down on a 300 μL drop of 3 % uranyl acetate for 10 min.

10. Wash ultrathin sections on grids twice with deionized water by floating grids section side down on a 300 μL drop of deionized water for 10 min.

11. Blot the bottom of the grid with Whatman #1 filter paper and place sample side upon a dry piece of filter paper in a Petri dish and allow to dry overnight.

12. Examine sections in the electron microscope. Figures 1 and 2 are examples of protein-A gold labeled immuno-electron micrographs of *Euglena* cells stained with antibodies to LHC II and the large subunit of RuBisCo.

RuBisCo dynamics in the *Euglena* cell cycle

Pyrenoid exists in the chloroplasts of most eukaryotic algae and moss plants, and have been considered as a mere storage place of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo), but its functions are still poorly understood now[9]. RuBisCo consist of a large subunit protein (MW : 55 kDa) encoded by chloroplast DNA and a small subunit (MW : 15 kDa) synthesized by nuclear DNA

in the cytoplasm, and is an important photosynthetic enzyme with a role in the first step of CO_2-fixation. Arrows in Fig. 2 indicates electron microscopy images of pyrenoids observed in the chloroplast of single-cell zooxanthella *Euglena*. Pyrenoids lie in the middle of the chloroplast, and the thylakoid membrane penetrates its substrate, and its peripheral area is surrounded by cytoplasmic paramylum (Fig. 2). The pyrenoid structure has no membrana limitans that divides peripheral chloroplast stroma, and is observed as a high-electron density structure (arrow in Fig. 2).

In our laboratory, we used immuno-electron microscopy as a first step to know the functions of *Euglena* cell pyrenoids, and traced structural changes of pyrenoids and molecular dynamics of RuBisCo proteins over time in the cell cycle of synchronized *Euglena* culture. As a result, Osafune *et al*. first discovered a close relationship between pyrenoids and CO_2-fixation in photosynthesis by immuno-electron microscopy[6, 10, 11]. Synchronized culture population of *Euglena* cells can

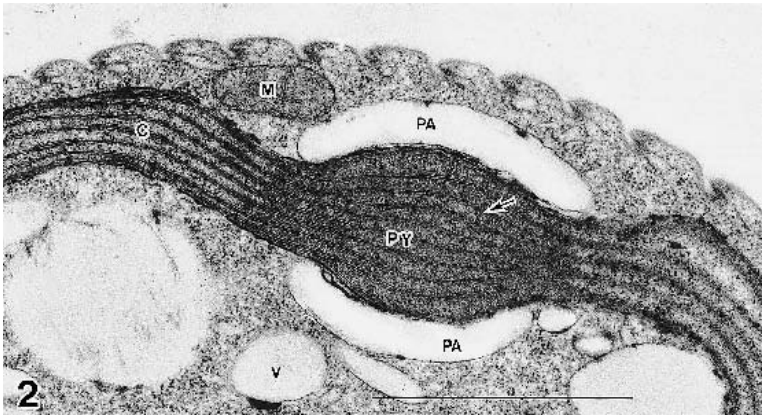

Fig. 2

A section of chloroplasts in a green *Euglena gracilis* Z cell. The arrow shows the pyrenoid. Bar : 1 μm. C : chloroplast, M : mitochondrion, PA : paramylum, PY : pyrenoid, V : vacuole.

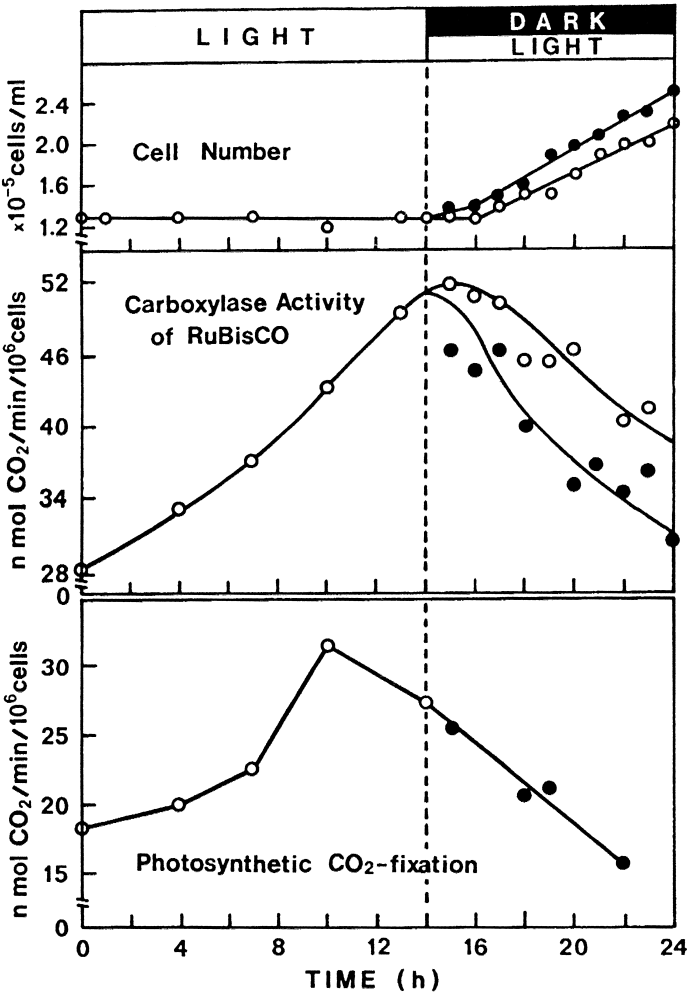

Fig. 3

Time course of changes in photosynthetic CO_2-fixation and the carboxylase activity of RuBisCo of *Euglena* cells during the cell cycle in synchronized culture. Staring cells had been synchronized under the 14 hr light : 10 hr dark regimen, and were placed under the same light : dark cycle (solid circles) as well as in continuous light (open circles) at 25℃. The same light intensity (6,000 lux) was used in synchronized cultures and that in measurements of photosynthetic CO_2 fixation. The enzyme activity was measured at 30℃.

easily be obtained under both inorganic photosynthetic conditions and heterotrophic conditions[6]. As shown in Fig. 3 (Upper), synchronized culture population of *Euglena* cells was obtained by 14 hr light : 10 hr dark regimen[6]. *Euglena* cells grow during the light period, and undergo binary division during the following 10 hours both in the dark and light exposure (Fig. 3 upper).

Figures 4-8 present pyrenoid structural changes traced using anti-RuBisCo antibody immuno-electron microscopy over time during the cell cycle of synchronized *Euglena* cell culture. Figure 4 presents chloroplasts in the 0-hr cells immediately after light exposure, and colloidal gold particles indicate RuBisCo localization. Slightly more RuBisCo molecules accumulated in the immature pyrenoid structure (arrow shows), compared with the stroma, can be observed (Fig. 4). Figure 5 shows chloroplasts at 13 hours of light exposure, indicating specific

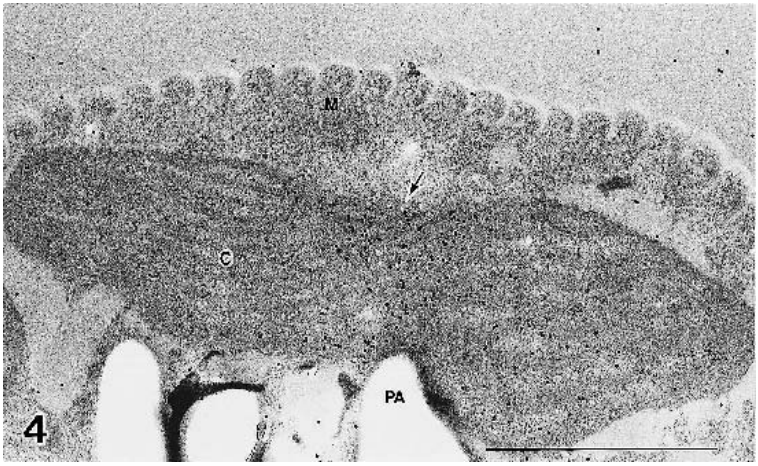

Fig. 4

A cell at the beginning of the light period (0-hr cell) labeled with anti-RuBisCo followed by protein A-gold. The arrow indicates the pyrenoid region, where gold particles are denser than in the rest of the chloroplast. C : chloroplast, M : mitochondrion, PA : paramylum. Bar : 1 μm.

localization of RuBisCo molecules on the developed pyrenoid structure. The pyrenoid structure during the cell cycle most highly developed at 13 hr, and at the same time, showed a highest photosynthetic activity level (Fig. 3 lower)[6]. More specifically, after serial ultrathin sections of the cells are treated with anti-RuBisCo antibodies, sequential immuno-electron microscopy images are obtained. Next, immuno-electron microscopy images are sequentially fed into the computer, and three-dimensional distribution of intracellular RuBisCo molecules and stereoimages of pyrenoids and thylakoid membranes were constructed[10]. According to the computer graphics analysis, volumes of the chloroplast and pyrenoid structure on this stage were about 94.6 μm^3 and 4 μm^3, respectively[10]. The computer graphics analysis was shown three-dimensional distribution of total amount of RuBisCo in the chloroplast. This shows RuBisCo is concentrated in the pyrenoid[10]. Based upon the above results, we first revealed that about 89% and 11% of the total amount of RuBisCo are localized in the pyrenoid and stroma areas, respectively[10].

Figure 3-middle and -upper show photosynthetic CO_2-fixation and carboxylase activity of extracted RuBisCo, traced over time during the cell cycle, respectively[6]. Carboxylase activity increased about twofold in the growth phase, and decreased in the mitotic phase both in the dark and light places (Fig. 3-middle). In addition, CO_2-fixation measured during photosynthesis reached the highest level around 10 hours after light exposure (6,000 lux), then began to reduce rapidly (Fig. 3-middle)[6]. However, when CO_2-fixation was measured with strong light (saturation), CO_2-fixation during photosynthesis reached above 100% of the total enzyme activity, and at the same time, developed pyrenoid structures were observed (Fig. 5). The pyrenoid structures disappeared on entering cell division phase, and at the same time, gold particles (RuBisCo) dispersed in the stroma along the thylakoid membrane (Fig. 6), and be scattered in the stroma after 21 hours[6]. Then it was demonstrated that immature pyrenoid structure began to form again in the central area of the

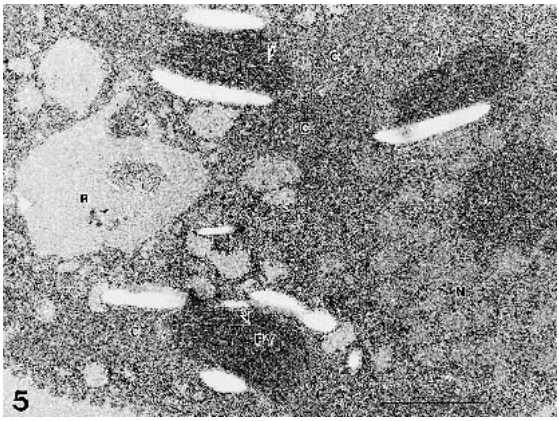

Fig. 5

A chloroplast in a 13 h-cell. The arrows indicate the pyrenoid, where gold particles are densely localized. C : chloroplast, N : nucleus, PY : pyrenoid, R : reservoir. Bar : 1 μm.

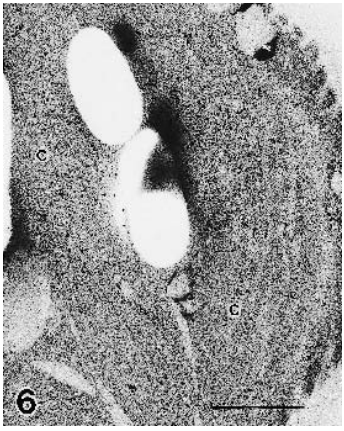

Fig. 6

A chloroplast in a 20 h-cell (6 h in the dark). Gold particles are localized in rows in the spaces between the thylakoids. C : chloroplast. Bar : 1 μm.

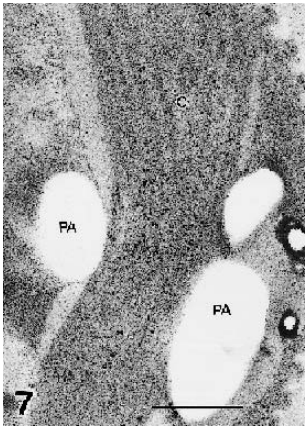

Fig. 7

The chloroplasts in a 24 h-cell. Gold particles are again concentrated in the center of the chloroplasts Note that the paramylum granules is present near the pyrenoid. C : chloroplast, PA : paramylum granule. Bar : 1 μm.

chloroplast at the end of division phase, and RuBisCo is also concentrated there (Fig. 7)[6].

In conclusion, RuBisCo involved in photosynthetic CO_2-fixation has been considered those dispersed in the chloroplast stroma until now, but based upon the dynamics of RuBisCo revealed by immuno-electron microscopy and measurement results of photosynthetic CO_2-fixation and carboxylase activity of RuBisCo presented in Fig. 3-middle-lower, it was first demonstrated that RuBisCo localized in the pyrenoid structure is likely to be involved in photosynthetic CO_2-fixation (Osafune *et al.* 1990)[6]. After that, McKay *et al.* revealed that RuBisCo actibase localizes in the mature pyrenoid structure by immuno-electron microscopy, supporting our findings that pyrenoid might be the photosynthetic site (McKay *et al.* 1991)[12].

LHC II proteins are transported to the chloroplast via Golgi apparatus.

Light-harvesting chlorophyll a/b binding protein complexes (LHC II) that harvest light to the photosystem are encoded by the cell nucleus and somehow transported to the chloroplast. It is known that they are incorporated into the thylakoid membrane, after undergoing two-step processing inside the chloroplast[13, 14]. Brandt *et al.*[15] investigated the LHC II incorporation into the thylakoid membrane over time using[14] C in the synchronized *Euglena* cell population by light-dark regimen. As a result, LHC II transport into the chloroplast thylakoid membrane started at about 6 hr after the initiation of light exposure, and showed a maximum level at 10 hr and declined till 18 hr. It has been reported that the transport stopped in 10 hr dark period.

We performed synchronized culture of *Euglena* cells by the same method of Brandt *et al.*,[15] and traced dynamics of LHC II protein molecules in the cell cycle by immuno-electron microscopy (Fig. 8)[16−18]. The solid line in Fig. 8 presents frequency of Golgi apparatuses with LHC II localized during the *Euglena* cell cycle

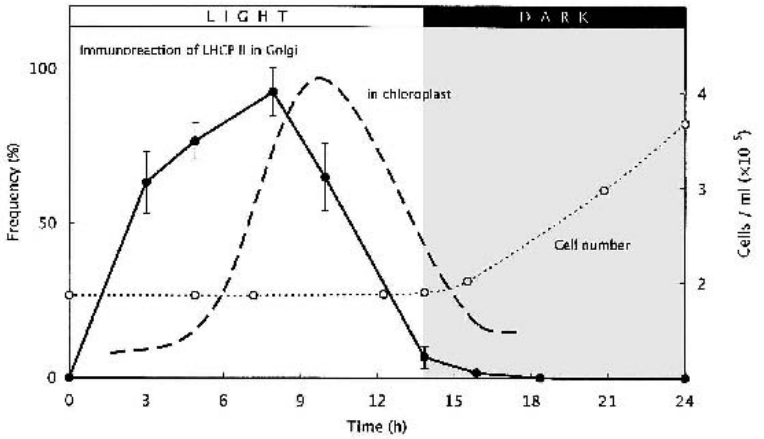

Fig. 8

Change in frequency of localization of LHC II proteins in the Golgi apparatus during the cell cycle of *Euglena gracilis* Z. Vertical bars represent the range of variation in three observations.

as a percentage[16]. In spite of the 2 hr time-lag, the solid frequency curve shows close similarity with the LHC II incorporation pattern into the thylakoid membrane, presented by Brandt *et al.*[15] More specifically, no LHC II localization in the Golgi apparatus was observed at 0 hr immediately after light exposure. At 3 hr after light exposure, LHC II localization was confirmed in 60% of the Golgi apparatuses, and it reached=90% at 8-10 hr. Subsequently, it showed sharp decline till 16 hr, and no localization in the Golgi apparatus was observed after 16 hr (Fig. 8). These results suggest that LHC II protein molecules synthesized in cytoplasm are transported to the thylakoid membrane via the Golgi apparatus[16, 17].

Next, the results observed by immuno-electron microscopy are shown (Figs. 9, 10). LHC II was observed on the chloroplast thylakoid membrane throughout the cell cycle[16]. In 0-hr cells immediately after division, LHC II was localized only on the thylakoid membrane, but not in the Golgi apparatus. Figure 9 is cells light-

exposed for 10 hours. They show that colloidal gold particles are localized both on the thylakoid membrane and in the Golgi apparatus that are pointed with the arrows[16]. Osafune *et al.* previously shown that specific localization of gold particles in electron-dense areas observed between the cisternae membranes and the cisternae in these Golgi apparatuses[16]. Three-dimensional distribution of LHC II was first reconstructed by our group first[18]. As revealed by computer graphics,

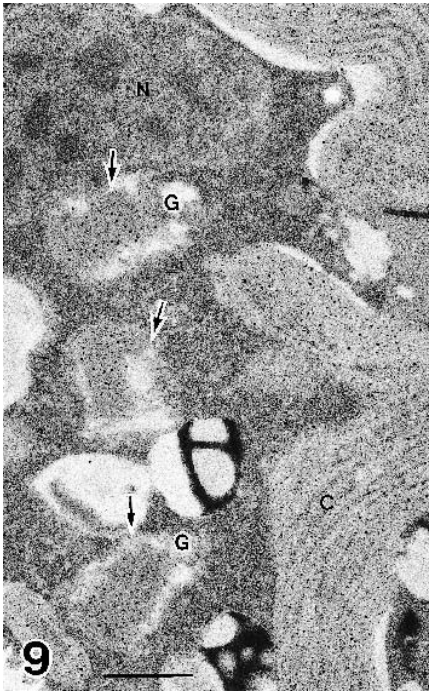

Fig. 9

A section in a 10 h-cell. Gold particles (LHC II proteins) are concentrated over Golgi (G : at the arrows) and thylakoids. C : chloroplast, G : Golgi apparatus, N : nucleus. Bar : 1 μm.

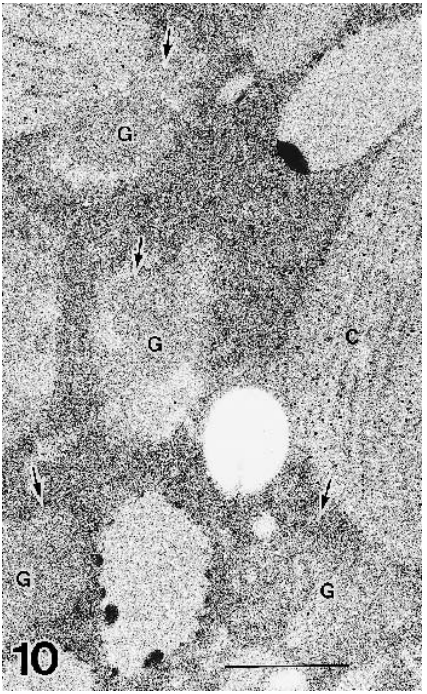

Fig. 10

A section of a 16 h-cell. Note that gold particles were localized over the thylakoids in chloroplasts, but not Golgi apparatus. The arrows indicate Golgi apparatus. C : chloroplast, G : Golgi apparatus. Bar : 1 μm.

LHC II protein molecules are localized in all the Golgi apparatuses and thylakoid membrane in 10-hr cells[18]. Figure 10 indicates 16-hr cells. As shown by the arrows, no LHC II localization in the Golgi apparatus is observed (fig. 10). Similarly, no localization of gold particles in the Golgi apparatus after 16 hr was observed.

As described above, dynamics of protein molecules in the cell cycle can be traced by using immuno-electron microscopy. We demonstrated that transport of cell nucleus-controlled LHC II protein molecules to the thylakoid membrane via Golgi apparatus could also be observed in the process of *Euglena* chloroplast formation[19-21]. This phenomenon that photosynthetic protein molecules are transported to the chloroplast via Golgi apparatus was first discovered by immuno-electron microscopy[21]. This finding is an entirely new phenomenon that protein molecules other than secretory proteins cross the Golgi apparatus[16, 17, 21].

In 1995, Prof. Steven Schwartzbach *et al.* of Nebraska State University, Department of Biochemically reconfirmed the phenomenon that we discovered by immuno-electron microscopy by tracing the transport route of LHC II protein molecules in *Euglena* cells with radioisotopes[22, 23]. After that, a research team led by Prof. Harvard Lyman of the New York State University reported that small sub-particles of RuBisCo enzyme encoded by cell nucleus were transported to the chloroplast via the Golgi apparatus in the same manner as LHC II (personal communication). The meaning of the temporary localization of LHC II protein molecules in the Golgi apparatus remains to be elucidated, while it is also speculated that a large precursor polypeptide (110-207 kDa) of synthesized LHC II might undergo processing in the Golgi apparatus (26.5 kDa) and transported into the chloroplast. Since this phenomenon observed in *Euglena* is also considered to be a common one observed in the transport of nucleus-controlled proteins to a specific organelle, other photosynthetic enzymes and mitochondria are under investigation.

Conclusions

It is well known that molecular biological approaches have had a high yield in the elucidation of life phenomena. However, it is also a fact that morphological approaches are indispensable in elucidating dynamics of whole biomolecules. Immuno-electron microscopy can trace intracellular molecule locations over time. In addition, combination of obtained immuno-electron microscopic images of serial ultrathin sections with computer graphics technology can elucidate three-dimensional distribution of intracellular protein molecules[10, 18]. However, as described above, intriguing subjects remain to be elucidated, such as from which and how most photosynthetic protein molecules of *Euglena* cells are transported to the chloroplast, and cross the chloroplast envelope with an *Euglena*-specific three-layer structure, and how these protein molecules are specifically incorporated into the chloroplast with a complex structure and express their functions[7, 24, 25]. To elucidate these problems, it will be necessary in the future to develop cell fixing agents and embedding resins, and integrate technologies such as immuno-electron microscopy and *in situ* hybridization method with molecular biology.

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