[Review]

Immunocytochemical Studies on the Behavior of Photosynthetic Proteins in Synchronized Cells of *Euglena gracilis* Z

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Changes in pyrenoid morphology and the distribution within the chloroplast of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) of *Euglena gracilis* Z were followed by immunoelectron microscopy during growth and division phase. Most of the immunoreactive protein was found in the pyrenoid during the growth phase with only a small amount of gold particles localized over the stroma. During the division phase, the pyrenoid was not detected and the gold particles were dispersed throughout the stroma. A comparison between rates of photosynthetic CO_2-fixation and the amount of total carboxylase activity catalyzed by RuBisCO extracted from *Euglena* cells in the growth phase suggests that pyrenoid localized RuBisCO is responsible for photosynthetic CO_2-fixation. The precursors to the *Euglena* light-harvesting chlorophyll *a/b* binding protein of photosystem II (LHCP II) are large polyproteins containing multiple copies of LHCP II covalently joined by a conserved decapeptide. Light induced LHCP II synthesis is controlled at the translational level in *Euglena* rather than the transcriptional level as found in higher plants and other algae. Under conditions promoting LHCP II synthesis and accumulation in the thylakoids, a reaction with anti-LHCP II antibody can be observed by immunogold electron microscopy in the Golgi apparatus. The kinetics of LHCP II appearance in the Golgi apparatus as measured by immunogold electron microscopy in synchronous cells and in cells undergoing normal light-induced chloroplast development suggests that nascent LHCP II is transported to the Golgi apparatus prior to chloroplast import and thylakoid insertion.

Key words: *Euglena*, Immunoelectron microscopy, Golgi apparatus, Ribulose-1,5-bisphosphate carboxylase/oxygenase, Light-harvesting chlorophyll *a/b* binding protein of photosystem II

I. Introduction

Recently, as structures of photosynthetic functional units, represented by chloroplast photosynthetic proteins, are being elucidated at the molecular level, dynamic states, transport, and complex formation mechanisms of these molecules have drawn attention[1, 28]. Mitochondrial and chloroplast DNAs were found about 40 years ago[2], 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.

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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
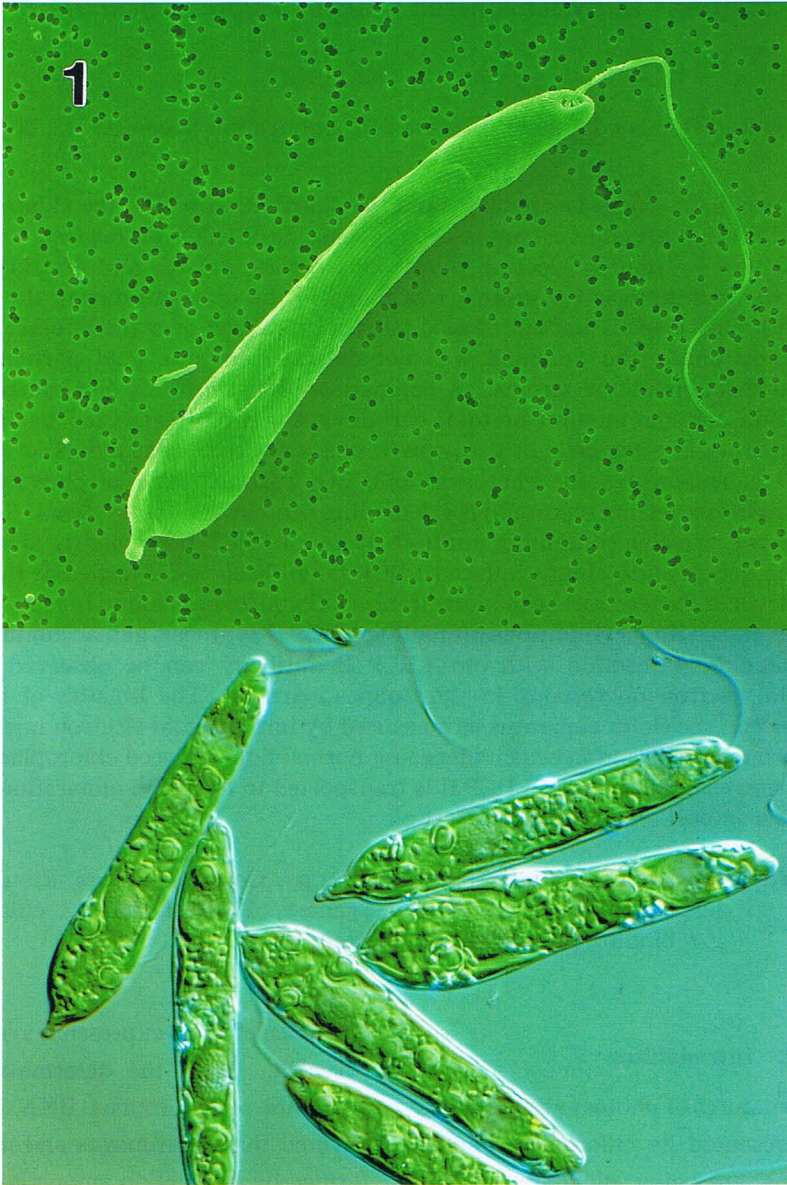

Fig. 1.   Scanning electron micrograph and light micrograph of *Euglena gracilis* Z cells.
Of the two flagella at the anterior of the cell of members of the Euglenales, only one emerges from the reservoir and canal (upper micrograph in Fig. 1). The pellicular strips, which converge at the cell extremities, are arranged in helical fashion, their overlapping giving the appearance of helical striae on the cell surface (upper micrograph). ×3,800.
Living *Euglena gracilis* Z cells observed under a light microscope (under micrograph in Fig. 1). Note that chloroplasts and the nucleus are clearly seen in sytoplasm using a light microsope. The nucleus is prominent in euglenoid cells and often readily visible in living individuals in the center or posterior of the cell. ×3,000.

antibody method in both resolution and contrast, and location of intracellular antigens can be comprehended with accuracy, since their location is replaced by high electron-density gold particles[3].

RuBisCO is the most abundant stromal chloroplast protein. Immunoelectron microscopy was used by Lacost-Royal and Gibbs[4] to study the distribution of RuBisCO in the pyrenoid and thylakoid region of the chloroplast of *Chlamydomonas reinhardtii*. They found about 40% of the total small subunit and 30% of the large subunit in the plastid localized in the thylakoid region, presumably in the stroma, with the remainder in the pyrenoid. Using immunoelectron microscopy of *Chlorella pyrenoidosa*, McKay and Gibbs[5] showed that in either light-limited or light-saturated cells, the pyrenoid was heavily labeled by antibody to each subunit of RuBisCO while chloroplast stromal labeling was not above background levels suggesting that the pyrenoid RuBisCO is enzymatically active *in vivo*. *Euglena* chloroplasts evolved through secondary endosymbiosis between a phagotrophic trypanosome host and eukaryotic algal endosymbiot (Fig. 1). In our laboratory, we found that the pyrenoid is absent in proplastids of dark-grown cells of *Euglena gracilis* Z[8]. When these cells were transferred to an inorganic medium, rudimentary pyrenoids were formed in the dark in developing plastids and fully developed pyrenoids appeared only after exposure of these cells to light[6-8]. Cook *et al.*[9] reported that the pyrenoid is found in the chloroplasts of synchronized cells of *Euglena gracilis* only in the first half of the light period. Kiss *et al.*[10] showed by immunofluorescence microscopy that the pyrenoid of *Euglena gracilis* is labeled by a RuBisCO antibody and that the appearance and disappearance of the pyrenoid under different nutritional conditions was related to the distribution of RuBisCO in the chloroplast.

The most abundant thylakoid protein is LHCP II. The primary apoprotein of *Euglena* LHCP II is a 26.5 kDa polypeptide[11] which is nuclear encoded and cytoplasmically translated[12-15] as in other systems. The precursor to *Euglena* LHCP II (pLHCP II) is an extremely large slowly processed polyprotein composed of 8 mature subunits linked by a conserved decapeptide[12, 15, 16]. A stromal protease releases mature LHCP II from the polyprotein precursor by removing the decapeptide[17]. A highly reactive specific polyclonal antibody to the 26.5 kDa LHCP II apoprotein[11, 14] has been used to monitor pLHCP II and mature LHCP II levels during chloroplast development[14, 15, 18]. When dark-grown resting cells of *Euglena* are exposed to light at the low intensity threshold of chloroplast development (7 ft-c), LHCP II can barely be detected. On exposure of these cells to the normal light intensities for chloroplast development (80–150 ft-c), the antenna proteins including the LHCP II apoprotein and chlorophyll *b*, rapidly accumulate[18]. Thin sections of cells at 7 ft-c do not show appreciable immunogold staining of either the Golgi apparatus or thylakoids with rabbit immune serum against the 26.5 kDa LHCP II apoprotein. When these cells are incubated at 80–150 ft-c, both the Golgi apparatus and thylakoids are immunogold labeled by anti-LHCP II[19-23]. The appearance of LHCP II in the Golgi apparatus first raised the possibility that the secretory pathway is utilized to transport proteins from the cytoplasm to the *Euglena* chloroplast.

As in *Euglena*, the pLHCP II of higher plants and most green algae is nuclear encoded and synthesized on cytoplasmic ribosomes[18]. The plant pLHCP II is post-translationally imported across the double envelope membrane of the plastid at contact sites formed through the interaction of the outer and inner membrane translocation system[24, 25]. The chloroplasts of higher plants and green algae are believed to have evolved through a singular primary en-

dosymbiotic event between a heterotrophic eukaryote and a cyanobacterial endosymbiont with the envelope membranes evolving from the host vacuolar membrane and the endosymbiont plasma membrane[26-28]. Upon transfer of genes from the endosymbiont to the host nucleus, a post-translational direct import system evolved to return the nuclear encoded cytoplasmically synthesized proteins to the evolving chloroplast.

Euglena chloroplasts have three envelope membranes. The additional envelope membrane is believed to be indicative of the evolution of the *Euglena* chloroplast through a secondary endosymbiotic association between a phagotrophic trypanosome host and a phototrophic eukaryote[7, 28]. The third outermost envelope membrane is thought to be derived from the host's phagocytic vacuolar membrane with the middle and innermost envelope membranes having evolved from the chloroplast envelope membranes of the eukaryotic endosymbiont[27, 28]. A unique cytoplasmic compartmentalized osmiophilic structure, the COS, found associated with the *Euglena* endoplasmic reticulum appears to be involved in the transport of some chloroplast proteins including the LHCP II to the chloroplast[29]. Taken together with the immunogold localization of LHCP II to the Golgi apparatus, this observation suggests that in *Euglena*, chloroplast proteins are translocated from the ER to the Golgi apparatus prior to chloroplast localization[30]. Schwartzbach and coworkers have utilized *in vivo* and *in vitro* experiments to show that the secretory system is utilized to transport *Euglena* chloroplast proteins from the cytoplasm to the chloroplast. LHCP II mRNA was associated with membrane bound rather than free polysomes[31]. Pulse chase subcellular localization studies demonstrated that pLHCP II and the precursor to the small subunit of RuBisCO (pSSU) were transported from the ER to the Golgi apparatus prior to chloroplast localization[31-34]. Surprisingly, the

precursors are transported as integral membrane proteins oriented with the presequence in the transport vesicle lumen and the mature protein on the cytoplasmic membrane face[34]. Lyman *et al.* have shown that translation of pLHCP II and pSSU are regulated by a light-induced second messenger system involving light induced Calcium release from the ER providing further evidence for involvement of the secretory system in the synthesis of light induced proteins[35]. When *Euglena* cells are synchronized by alternating 14 h-light and 10 h-dark cycles[36], the spherical or ovoid daughter cells produced from mother cells during the dark period start to grow photosynthetically when transferred to light (0 h). The cells growing in the light are initially elongated and gradually change to spherical cells. At the end of the 14 h light period, the cells transferred to the dark undergo first nuclear and then cellular division[36]. Chloroplast division precedes cell division. Immunoelectron microscopy was used to follow the distinct changes in pyrenoid morphology, the distribution of RuBisCO in the chloroplasts, and LHCP II in the thylakoids that occur during the cell cycle in synchronized *Euglena gracilis*.

II. Immunogold Localization of RuBisCO with Reference to Pyrenoid Morphology

Synchronized *Euglena* cells grew during the 14 h light period ('growth phase') and divided during the following 10 h period either in the dark or in the light ('division phase'), as seen from Fig. 2 (upper panel)[37]. Relative to cells placed in the dark, cell division was delayed by a few hours in cells remaining in the light. RuBisCO activity per cell in extracts prepared from synchronized cells increased nearly two fold during the growth phase and decreased during the division phase (Fig. 2, middle panel). The rate of photosynthetic CO_2-fixation also increased during the initial several hours in the light attaining the maximum level at about 10 h
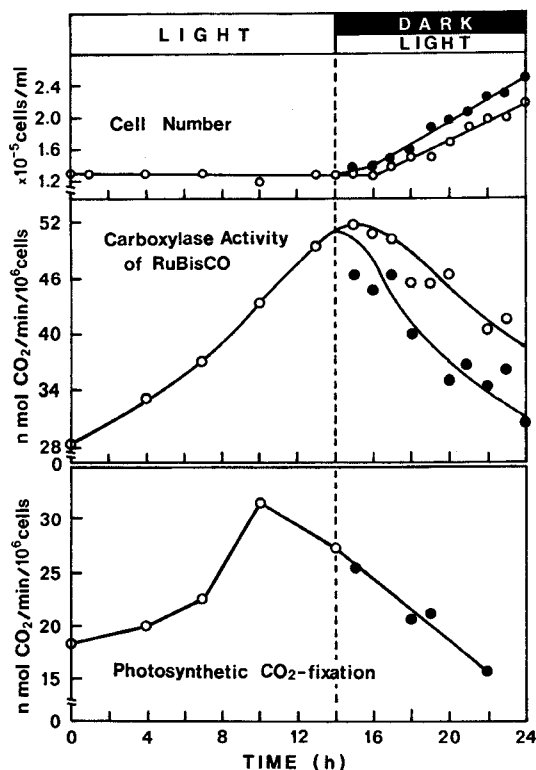
Fig. 2. Time course of changes in photosynthetic CO_2-fixation and the carboxylase activity of RuBisCO during the cell cycle in synchronized *Euglena*. *Euglena* cells were synchronized by an alternating 14 h : 10 h light dark cycle and maintained under an alternating 14 h : 10 h light dark cycle (solid circles) or placed under continuous illumination (open circles) at 25°C. The same light intensity (6,000 lux) was used for cell growth and for measurements of photosynthetic CO_2 fixation. RuBisCO carboxylation activity was measured at 30°C.

gradually decreasing in the dark or the light (Fig. 2, lower panel)[37].

The chloroplasts contained rudimentary pyrenoids at the beginning of the light period[7]. Anti-RuBisCO protein A-gold labeled sections contained gold particles more concentrated in the pyrenoid region than in other areas of the chloroplasts (Fig. 3). The rudimentary pyrenoids rapidly changed into typical pyrenoids with adjacent paramylum granules and the gold parti-

cles were markedly concentrated over the pyrenoid region[7]. Chloroplast division begins soon after the start of cell division[37]. Shortly before the chloroplasts start to divide, the pyrenoid begins to disappear. At this stage, immunogold labeling of the pyrenoid is reduced with a disperse labeling of the stroma. The pyrenoid was totally undetectable in the chloroplasts during the division phase of the cell cycle either in the dark or in the light and immunogold labeling indicated RuBisCO was completely dispersed to the stroma[37, 38]. Near the end of the division phase in the dark or the light, gold particles were found in the chloroplasts arranged in short lines in the narrow area of stroma between thylakoids, either in the dark or in the light (Fig. 4). Gold particles were subsequently found to be clustered in the center of chloroplast, concurrently with the reappearance of the rudimentary pyrenoid[37].

Examination of RuBisCO labeled immuno-electron micrographs of serial sections through the chloroplasts of actively growing cells revealed small areas distinctly enriched in gold particles as well as the label on the main pyrenoid region. These areas called 'satellite pyrenoids', could not be detected in division phase cells[37]. Table 1 shows the relative distribution of gold particles among the main pyrenoid, satellite pyrenoids and the stroma in four chloroplasts of a serially sectioned 10-h cell.

The pyrenoid is the site in algae where RuBisCO, the vital enzyme of the photosynthetic carbon-fixation cycle, is concentrated but it is not known whether RuBisCO in the pyrenoid is functional or is simply being stored[36]. The data in Fig. 2 show that the maximum CO_2-fixation measured at 25°C in 10-h cells grown under 6,000 lux was nearly the same as the total activity of RuBisCO extracted from the cells at the same stage[34] and measured at 30°C. According to the result of Yokota *et al.*[40], the RuBisCO activity at 25°C, the temperature at which the

3

Fig. 4. A cell labeled with anti-RuBisCO antibody
Euglena cells were synchronized by an alternating 14 h : 10 h light dark cycle. A section through chloroplasts of a 20-h cell (6 h in the dark) labeled with anti-RuBisCO followed by protein A-gold (×17,000). Gold particles are localized in rows in the spaces between the thylakoids.

Table 1. Relative Distribution of RuBisCO-Gold Particles among the Stroma and Pyrenoid Regions in Chloroplasts of a Cell from the Growth Phase Culture

| Chloroplast | No. of gold particles in the stroma | No. of gold particles in pyrenoids | | | | | | | | Sum total |
| | | Main | Satellite | | | | | | Total | |
			1	2	3	4	5	6		
1	339	1,451	300	23	18				1,792	2,131
2	307	1,780	37	18	13				1,848	2,155
3	187	969							969	1,156
4	316	1,005	551	46	22	18	16	12	1,670	1,986

The cell sample was taken from a synchronized culture at the 10th h from the beginning of the light period. Gold particles were counted in each of 41 serial sections of the cell and were summed.

Fig. 3. A cell labeled with anti-RuBisCO antibody
Euglena cells were synchronized by an alternating 14 h : 10 h light dark cycle.
A chloroplast from a 10-h cell labeled with anti-RuBisCO followed by protein A-gold (×23,000). Gold particles are densely localized over the pyrenoid. Note that paramylum (PA) is present surrounding the chloroplast at the pyrenoid region.
Abbreviations used here and on subsequent pictures: C, chloroplast; COS, compartmentalized osmiophilic structure; ER, endoplasmic reticulum; G, Golgi apparatus; M, mitochondrion; N, nucleus; PA, paramylum; PY, pyrenoid.

cellular CO_2-fixation was determined, can be estimated to be 75% of the activity measured at 30°C. When photosynthetic CO_2-fixation was measured at higher light intensities (10,000–20,000 lux), the maximum fixation rate was more than 100% of the total cellular enzyme activity (unpublished results). The pyrenoid was found at these higher light intensities in the chloroplasts of growing cells. These results strongly suggest that RuBisCO in the pyrenoid is enzymatically active in photosynthetic CO_2-fixation.

Higher plant chloroplasts contain inclusions which have been proposed to be related to the algal pyrenoid. Griffiths[39] has traced a gradual progression within the plant kingdom from the massive single algal pyrenoid to smaller multiple pyrenoids and finally to the very small proteinaceous areas distributed throughout the higher plant chloroplast. The pyrenoid of the liverwort *Anthoceros* is reported to consist of small units (25 to many hundreds) which may form an aggregated central mass within the chloroplast or which may be distributed throughout the chloroplast as small fragments[41-43]. Kaja[44] and Burr[45] described the possible evolutionary steps in pyrenoid disintegration in a comparison of different species of *Anthoceros*, which show a range of forms from a homogenous pyrenoid to complete dispersal throughout the chloroplast. The different morphologies of the *Euglena* pyrenoid, including satellite pyrenoids, observed during the cell cycle appears to be analogous to the different morphologies of the pyrenoid in the liverwort.

Dispersion of the pyrenoid in *Euglena* chloroplasts begins shortly before the start of chloroplast division in light:dark synchronized cells. Chloroplast division begins shortly after the start of cell division[38]. The dispersion of the pyrenoid might facilitate distribution of RuBisCO between daughter chloroplasts during chloroplast division. According to Cook *et al.*[9],

the pyrenoid is present only in the first half of the light period in light:dark synchronized *Euglena* cells. Under the conditions used by Cook, chloroplast division took place slightly before cell division[46]. Presumably, the pyrenoid had dispersed and become undetectable prior to the start of chloroplast division. Kiss *et al.*[10] used transmission electron microscopy and immunofluorescence microscopy to demonstrate that the appearance and disappearance of pyrenoids occurring concomitant with the concentration and dispersion of RuBisCO in *Euglena* chloroplasts was nutritionally controlled. Low nutrient levels were related to the presence of pyrenoids while high nutrient levels were related to the absence of pyrenoids. Orcival-Lafont and Calvayrac[47] also found a similar correlation between nutrient levels and the presence or absence of pyrenoids. Miyachi *et al.* reported that the pyrenoid is affected by environmental CO_2 concentrations[48]. The pyrenoids and the starch sheaths around the pyrenoids develop much more slowly in low-CO_2 cells than in high CO_2-cells of *Chlorella* and *Scenedesmus*. We observed in *Euglena* that pyrenoid formation occurs concomitantly with RuBisCO accumulation over the pyrenoid in the proplastids of dark-organotrophically grown *Euglena gracilis* Z cells transferred to an inorganic medium in the dark[49-51].

## III. Stage-dependent Localization of LHCP II in Synchronized Cells of *Euglena*

Immunoelectron microscopy using protein A-gold and specific rabbit antibody against the 26.5 kDa LHCP II apoprotein[11, 14], the principal polypeptide of the *Euglena* LHCP II light harvesting *a/b* complex[11], was used to follow the synthesis of LHCP II during the cell cycle. Cells of *Euglena gracilis* strain Z were synchronized by 14 h light 10 h dark periods. Samples for immunoelectron microscopy were taken at the beginning of the light period and

sampling continued throughout the 14 h light period and the 10 h dark period concluding at 24 h. It should be noted that the 0 h cells and 24 h cells are newly divided cells differing by one generation. Cell number remained constant throughout the light period and cell number doubled during the dark period as seen from Fig. 2 (upper panel)[37].

The end of the dark period and beginning of the light period, gold particles are concentrated over the thylakoids but few are found over the Golgi apparatus indicating that LHCP II apoprotein is present only in the thylakoids when cells have completed dividing in the dark. The synthesis of LHCP II is light dependent and is triggered at 0 h when the cells are illuminated. LHCP II apoprotein, are present over the compartmentalized osmiophilic body (COS) at 1 h. This structure is composed of a central osmiophilic core with reticulate extensions of the same material surrounding cytoplasmic material including ribosomes[29] and is found in various cells of *Euglena gracilis* and mutants[29]. At 5 h, gold particles are concentrated over the Golgi apparatus and thylakoids indicating the presence of LHCP II apoprotein in both compartments. As a control, a similar section was treated with preimmune, rather than immune serum. There are very few gold particles and these are distributed randomly over the section. Thus the immunogold reaction over the Golgi apparatus is indicative of the presence of LHCP II and is not due to a nonspecific immunoreaction. After 10 h in the light (Fig. 5), gold particles are concentrated over thylakoids and the Golgi apparatus. Localization to the Golgi apparatus is seen more clearly at high magnification using smaller gold particles where the antibody reaction can be seen to be associated with both the Golgi cisternae and the dense material that lies between them. To ensure that this distribution is not a fixation artifact, the same samples were prepared by the freeze-substitution method

and an identical distribution was seen[42]. Gold particles are also observed over the COS in 10 h cells. The synthesis of LHCP II is light dependent ceasing upon the onset of the dark period. At 23 h, gold label was localized over the thylakoids of the daughter chloroplasts (Fig. 6). The Golgi apparatus was not immunogold labeled in the 23 h cells which were not actively synthesizing LHCP II as seen from Fig. 6. A quantitative analysis of the frequency of Golgi apparatus immunogold labeling during the cell cycle was reported by Osafune *et al.*[52]. The frequency of Golgi immunogold labeling is defined as the number of immunogold labeled Golgi observed over the total number of Golgi The Golgi apparatus is not immunogold labeled at the beginning of the light period. Coincident with the onset of light induced LHCP II synthesis, the frequency of immunogold labeling of the Golgi apparatus increases during the first 8 h of the light period and then declines[52]. The beginning of the dark period, the Golgi apparatus is not immunogold labeled and the Golgi apparatus remains unlabeled throughout the remainder of the dark period[52]. As expected, the thylakoids are always immunogold labeled (Fig. 6). A similar pattern of Golgi apparatus immunogold labeling is found in synchronized cells of the *bacillaris* strain[52]. As with the Z strain, both the Golgi apparatus and the thylakoids are stained with anti-LHCP II-immunogold during the light period but not the dark period[52, 53]. Synchronized cells of the *bacillaris* mutant Gr₁BSL which lacks the LHCP II apoprotein[11] do not show staining of either the Golgi apparatus or thylakoids at any growth stage[52].

We have used a highly specific and reactive antibody against the 26.5-kDa LHCP II apoprotein along with protein A-gold to localize the apoprotein in electron micrographs of synchronous cells of *Euglena*[52]. As might be expected, the apoprotein is found at all cell cycle stages in the chloroplast thylakoids of wild-type

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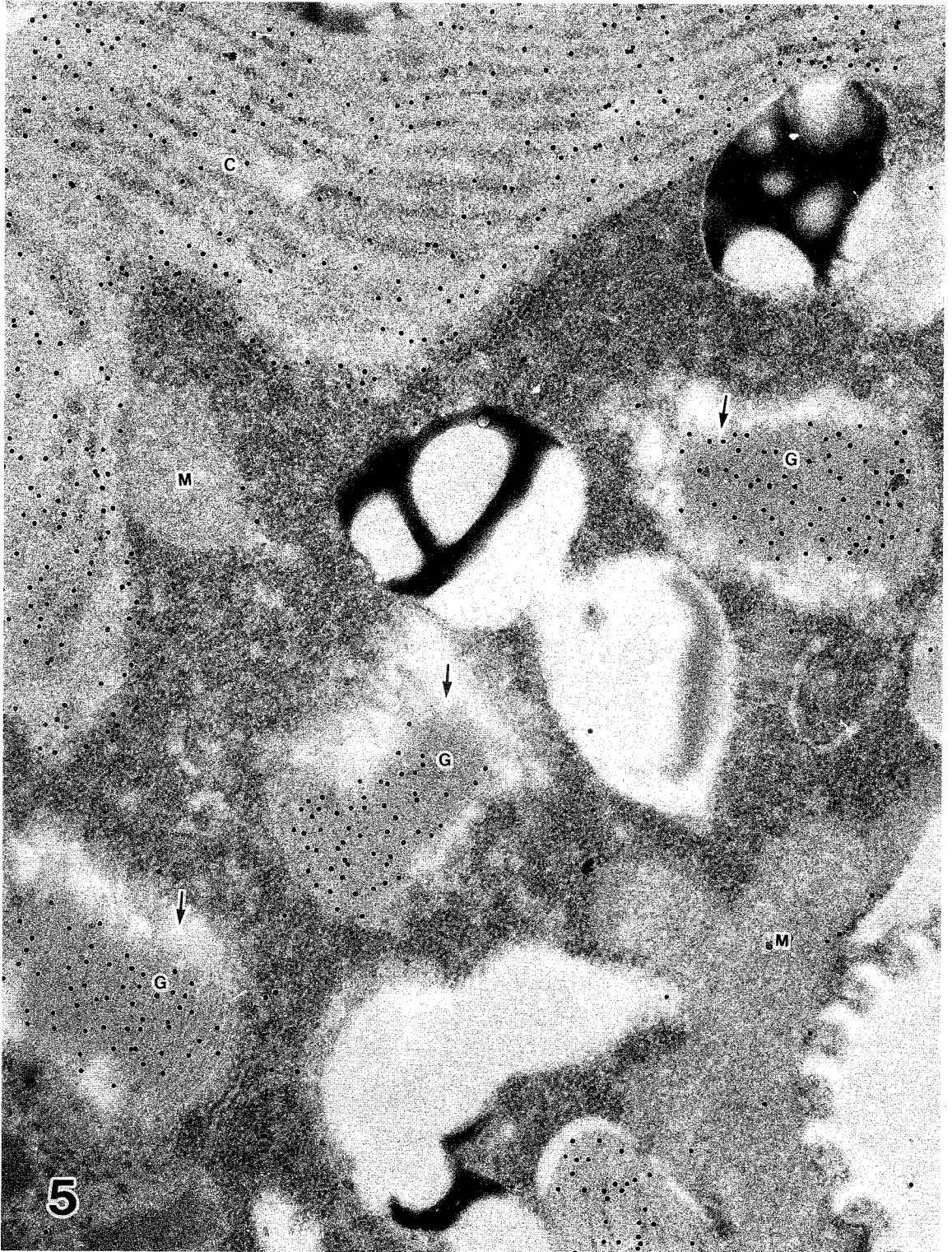
cells of the *bacillaris* and Z strain. The apoprotein is absent from the Golgi apparatus at the beginning of the light period. The frequency of Golgi apparatus immunolabeling reaches a peak at 8 h of the light period and declines so that by 16 h, Golgi apparatus labeling is undetectable. Brandt and Von Kessel[54] studied the incorporation of ^{14}C amino acids into thylakoid LHCP II in the Z strain during the cell cycle. They found little incorporation during the first 6 h into the light period. Peak incorporation was observed at 10 h falling to a low incorporation rate by 18 h with little incorporation at 24 h, the end of the dark period. The immunogold labeling of the Golgi apparatus which we observed started earlier, rises more gradually and falls more rapidly than the thylakoid labeling seen by Brandt and von Kessel[54]. This is the pattern expected if newly synthesized LHCP II is transported through the Golgi apparatus prior to appearance in the thylakoids. Assuming a limited and regulated source of LHCP II apoprotein is produced from the cytoplasmic ribosomes, this "pulse" of apoprotein would transiently appear in the Golgi before joining the accumulated apoprotein in the thylakoids. Synchronized cells of Gr$_1$BSL, a mutant of *bacillaris* lacking the 26.5-kDa LHCP II apoprotein, did not show staining of thylakoids or Golgi at any stage, indicating that the immunoreaction is specific for the LHCP II apoprotein.

IV. Photosynthetic Protein Transfer from the COS to the Golgi Apparatus

The few views of the compartmentalized osmiophilic body (COS)[29] that we have obtained indicate the presence of LHCP II apoprotein. It is not possible to provide systematic data for this structure throughout the cell cycle since it is small and difficult to find. Further experiments are required to clarify the nature of the COS and its role in LHCP II synthesis. The fact that the COS showed the same staining with anti-LHCP II antibody at 1 h (when Golgi staining was negligible) and at 10 h (when Golgi staining was high) suggests that LHCP II may be present in the COS at all stages when LHCP II is being synthesized (Fig. 7). As we have previously noted[29, 58, 59], the COS has compartments which enclose portions of cytoplasm including ribosomes and it is always close to the endoplasmic reticulum (Fig. 8-A and -B, an arrow shows)[29]. More recent studies indicate that the COS may be attached to the endoplasmic reticulum[55, 59]. It is possible, then, that the COS is a specialized region of the rough endoplasmic reticulum where LHCP II messages are translated with LHCP II passing into the ER for subsequent transport to the Golgi apparatus. LHCP II mRNA is associated with membrane bound polysomes and light induced LHCP II synthesis is under translational control at the level of elongation in *Euglena*[13, 31, 57, 58]. If future work shows that the COS is uniformly stained throughout the cell cycle including times when LHCP II is not being actively synthesized, this would suggest that nascent LHCP II chains are present in the COS but translation on the COS associated ribosomes is arrested. Photo-induction of LHCP II synthesis may result from the light dependent release of this elongation arrest allowing LHCP II synthesis on COS associated ribosomes.

V. *Euglena* Golgi to Chloroplast Transport Is Reversibly Blocked by Incubation at 15℃

Reduced temperatures reversibly disrupt

Fig. 5. A cell labeled with anti-LHCP II antibody
  Euglena cells were synchronized by an alternating 14 h : 10 h light dark cycle. A section through the 10 h-cell labeled with anti-LHCP II followed by protein A-gold. Gold particles are concentrated over the Golgi apparatus (arrows) and thylakoids. ×27,000.

transport along the exocytic and endocytic pathway in a variety of cells. *Euglena* pLHCP IIs (precursor) are a group of 207, 166, 122 and 110 kDa polyproteins which are cleaved by a chloroplast localized processing peptidase to produce mature LHCP IIs[15, 17].
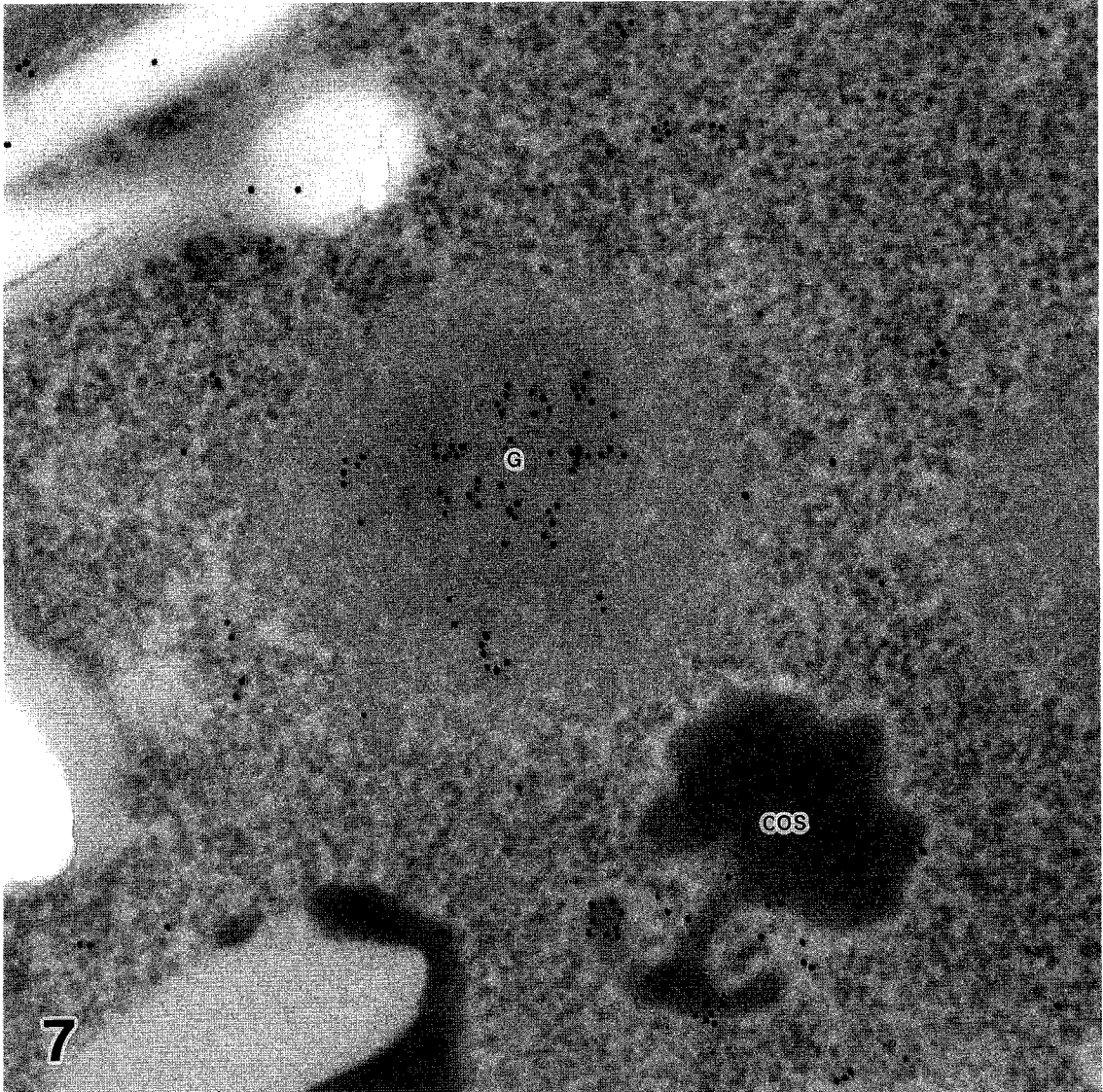

Fig. 7. A cell labeled with anti-LHCP II antibody
Euglena cells were synchronized by an alternating 14 h : 10 h light dark cycle. A section through the COS in a 1 h-cell labeled with anti-LHCP II followed by protein A-gold. Gold particles are present over the COS and Golgi. ×90,000.

Fig. 6. A cell labeled with anti-LHCP II antibody
Euglena cells were synchronized by an alternating 14 h : 10 h light dark cycle. A low magnification of a 23 h-cell labeled with anti-LHCP II followed by protein A-gold. Note that gold particles are localized over the thylakoids but not the Golgi apparatus (arrows) in daughter chloroplasts. ×18,000.
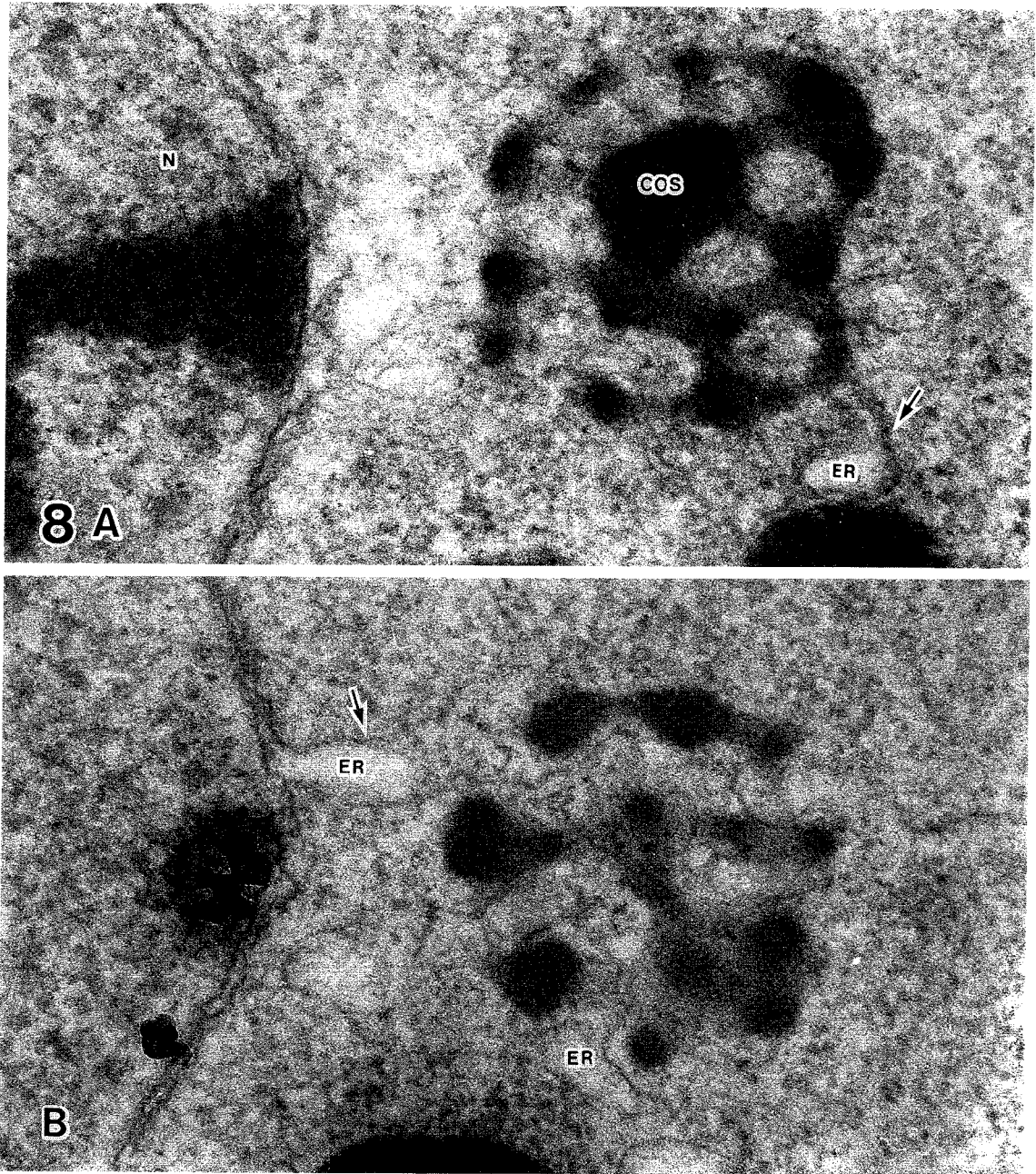
Fig. 8-A and B. Consecutive serial cell sections of *Euglena* showing the structure of compartmentalized
osmiophilic body (COS)
As we have previously noted (29), the COS has compartments which enclose portions of
cytoplasm including ribosomes and it is always close to the endoplasmic reticulum (an
arrow shows). More recent studies indicate that the COS may be attached to the
endoplasmic reticulum. ×90,000.

10 MINUTE PULSE

%TOTAL RECOVERED

10 MINUTE PULSE 15°C, 20 MINUTE CHASE

10 MINUTE PULSE 15 °C, 30 MINUTE CHASE

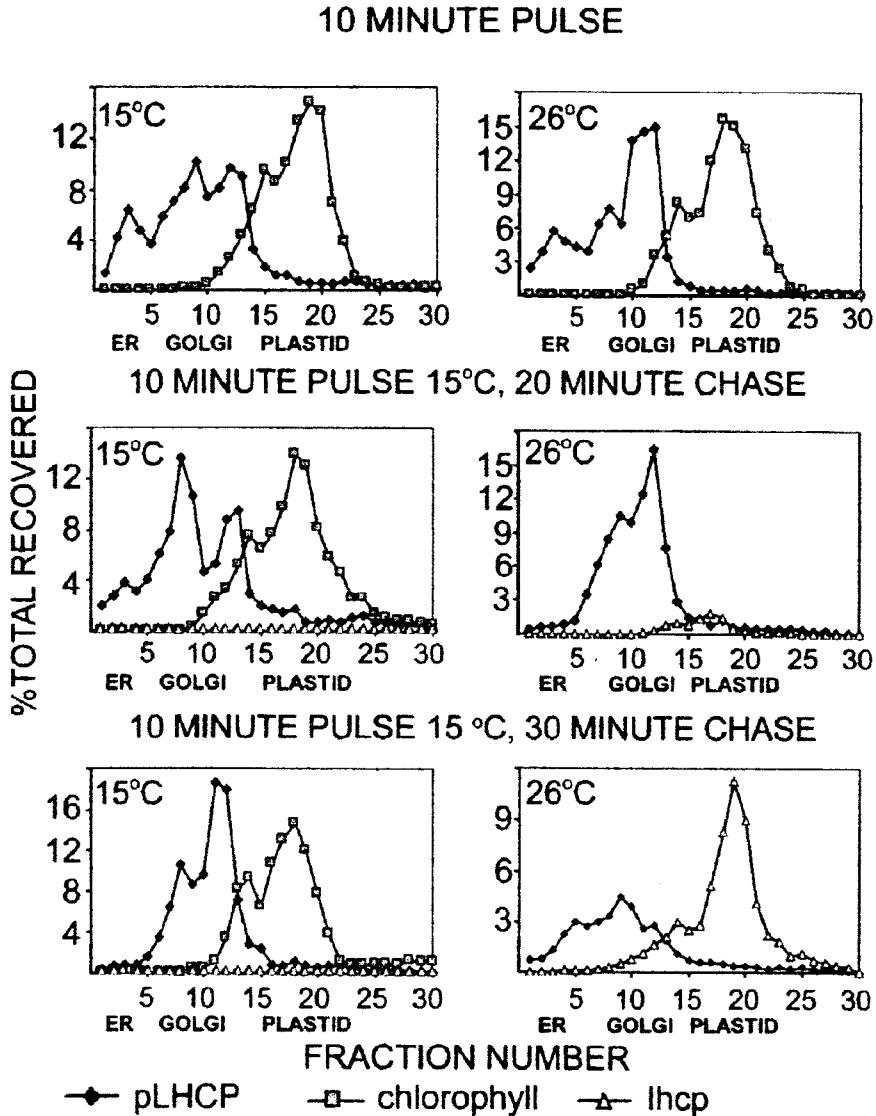## FRACTION NUMBER

—◆— pLHCP   —□— chlorophyll   —△— lhcp

Fig. 9. Incubation at 15℃ reversibly inhibits transport of pLHCP II from the Golgi apparatus to the chloroplast. Cell free extracts were prepared from dark-grown *Euglena* exposed to light at 26℃ for 24 h, incubated at 15℃ or 26℃ for 2 hours and pulse labeled at 15℃ or 26℃ for 10–29 minutes with [35S]-sulfate. The cells pulse labeled at 15℃ were chased for 20 or 30 minutes at 15℃ or 26℃ with unlabeled sulfate. Organelles were separated by isopycnic sucrose gradient centrifugation, and each gradient fraction was immunoprecipitated with an antibody raised against *Euglena* LHCP II. Immunoprecipitates were analyzed on SDS gels, and the gels were scanned with a PhosphorImager. To allow direct comparisons between gradients loaded with differing amounts of [35S] sulfate-labeled protein, the amount of pLHCP II and LHCP II in each fraction is plotted as a percentage of the total immunoprecipitate (pLHCP II and LHCP II) recovered from the gradient.   pLHCP II accumulated in the Golgi apparatus in cells maintained at 15℃ and was transferred to the chloroplast and converted to mature LHCP II between 20–30 minutes after transfer to 26℃.

Preliminary experiments showed that when dark grown resting *Euglena* were exposed to light at 26℃ for 24 hours, incubated at 15℃ for two hours, pulse labeled with $H_2[^{35}S]O_4$ for 10 min at 15℃ and chased for up to 40 min at 15℃, the pLHCP II polyproteins were the predominate proteins immunoprecipitated. This contrasts with cells chased at 26℃ where virtually complete conversion of pLHCP II to LHCP II occurred between 20–30 min of the chase suggesting that 15℃ blocks pLHCP II transport to the chloroplast where it is rapidly converted to LHCP II[15, 17]. To determine if 15℃ blocked a specific step in the ER to Golgi to chloroplast vesicular transport pathway, cells were pulse labeled at 15℃, chased at 15℃ or 26℃ and at appropriate times, the intracellular localization of pLHCP II and LHCP II was determined by immunoprecipitation of subcellular fractions separated on sucrose gradients. Marker enzymes identified fractions 2–5 as containing ER membranes, fractions 6–14 as containing light and dense Golgi membranes and fractions 14–22 as containing broken and intact chloroplasts[14, 15, 17]. For direct comparisons between gradients loaded with differing amounts of ^{35}S-labeled protein, the amount of pLHCP II and LHCP II in each fraction is plotted as the % of total immunoprecipitate (pLHCP II and LHCP II) recovered from the gradient. Chlorophyll in each fraction plotted as % total chlorophyll recovered provides an internal standard for the position of broken (first chlorophyll peak) and intact chloroplasts (Fig. 9)[1].

After a 10 min pulse at 15℃ or 26℃, pLHCP II is found predominately in the Golgi containing fractions with a smaller but significant amount in the ER (Fig. 9)[1]. After a 10 min pulse at 15℃ and a 20 or 30 min chase at 15℃, the amount of ER localized pLHCP II decreased with a concomitant increase in the amount of pLHCP II in Golgi containing fractions. In the experiment presented in Fig. 9, Golgi fractions contained after a

20 min chase 75% and after a 30 min chase 90% of the total pLHCP II immunoprecipitate recovered demonstrating pLHCP II transport from the ER to the Golgi apparatus at 15℃[1]. In cells pulsed for 10 min at 15℃ and chased for 20 min at 26℃, the amount of pLHCP II in Golgi containing fractions increased to 80% of the total immunoprecipitate recovered (Fig. 9)[1]. In contrast to cells chased at 15℃ where LHCP II was never found in the plastids, a small but significant amount of LHCP II, 9% of the total immunoprecipitate recovered, was present in chloroplasts after a 20 min chase at 26℃. At the end of a 30 min chase at 26℃, pLHCP II in Golgi fractions accounted for 25% of the total immunoprecipitate while chloroplast localized LHCP II accounted for 60% of the immunoprecipitate (Fig. 9)[1]. pLHCP II was found in Golgi fractions and not in chloroplasts while LHCP II was confined to chloroplasts and never in the Golgi apparatus indicative of pLHCP II transport to the chloroplast at 26℃ and rapid processing of the polyprotein to LHCP II by a chloroplast protease[16]. This contrasts to the absence of accumulation of pLHCP II or LHCP II in chloroplasts of cells chased at 15℃ clearly indicating Golgi to chloroplast transport is blocked by incubation at 15℃.

VI. Fusion of Vesicles to the Chloroplast Envelope Visualized by Electron Microscopy

Euglena cells were fixed with 2% glutaraldehyde at 4℃, post-fixed with 1% osmium tetraoxide for 60 min at 4℃, and embedded in agarose. After dehydration in an ethanol series followed by acetone, cells were embedded in Spurr's resin. Thin sections were cut with a diamond knife. Sections were post-stained with 3% uranyl acetate followed by lead citrate, and examined with a JEOL 100CX Electron Microscope. The first step in *Euglena* chloroplast protein import is the fusion of Golgi transport vesi-
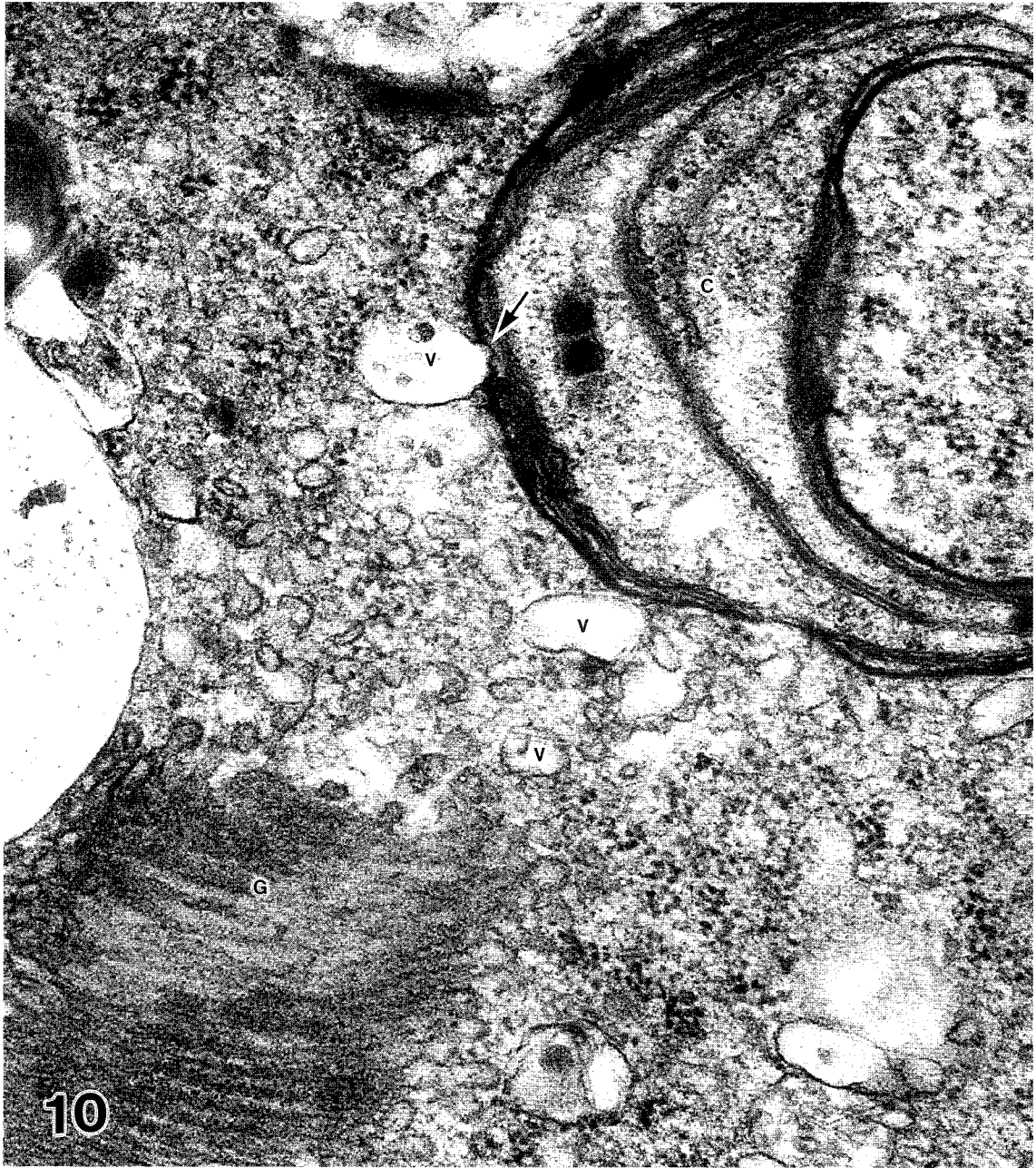
Fig. 10. Numerous vesicles are observed between the Golgi apparatus and chloroplast
          A number of vesicles are seen adjacent to the chloroplast and the membrane of one of these
          vesicular structures is clearly continuous with the chloroplast envelope indicative of vesicle
          chloroplast fusion (the arrows show). ×90,000.

cle with the chloroplast. pLHCP II accumulates in the Golgi apparatus of cells incubated at 15℃ for 2 h and transport to the chloroplast resumes by 20 min after return to 26℃[1]. The three chloroplast membranes are tightly appressed and seen in electron microgaphs as a single entity

rather than three separate membranes in 15℃ cells returned to 26℃ for 20 min. Numerous vesicles are observed between the Golgi apparatus and chloroplast (Fig. 10). A number of vesicles are seen adjacent to the chloroplast and the membrane of one of these vesicular structures is clearly continuous with the chloroplast envelope indicative of vesicle chloroplast fusion (Fig. 10 arrows). Vesicles are not normally seen fusing to chloroplasts of cells developing continuously at 26℃ suggesting that release of the 15℃ temperature block produced a transient increase in vesicular traffick between the Golgi apparatus and chloroplast.

VII. Conclusions

The immunogold localization of LHCP II apoprotein to the Golgi apparatus only at times of active synthesis suggests that LHCP II is transported to the Golgi apparatus prior to import into the chloroplast and localization to the thylakoids. We have treated sections of *Euglena* with specific active antibody against the large and small subunits of RUBP carboxylase/oxygenase and find no staining of the Golgi under a variety of conditions (data not shown). Pulse chase subcellular fractionation studies have however found both LHCP II and RuBisCO are transported from the ER to the Golgi apparatus prior to chloroplast localization[34, 57-60]. The precursors to both *Euglena* LHCP II and the small subunit of RuBisCO are extremely large polyproteins composed of multiple units of the mature protein covalently linked by a conserved decapeptide[12, 15, 16, 56]. The polyprotein processing protease is a stromal enzyme[17] allowing the appearance of mature protein to be used as an indicator of polyprotein delivery to the chloroplast. The half life of the LHCP II precursor is 20 min while that of the small subunit precursor is 10 min[32, 33] indicating that the small subunit of RuBisCO is transported from its site of synthesis in the cytoplasm to the chloroplast at twice the

rate of the LHCP II precursor. Thus the failure to detect small subunit in the Golgi apparatus is not indicative of a failure to transport small subunit to the Golgi apparatus prior to chloroplast localization but rather a reflection of the fact that due to its high rate of transport, the level of small subunit precursor in the Golgi apparatus is probably below the limits of detection by immunomicroscopy.

Euglena chloroplasts are believed to have evolved through a secondary endosymbiotic association between a phagocytic trypanosome host and a eukaryotic algae[27, 28]. The *Euglena* chloroplast evolved within the phagocytic vacuole of the host. Upon transfer of genes for chloroplast proteins from the endosymbiont to the host nucleus, a mechanism was needed to return the proteins from their cytoplasmic site of synthesis to the chloroplast residing within the host's phagocytic vacuole. Vacuolar proteins are co-translationally transported into the ER, transported to the Golgi apparatus and then sorted into vesicles for transport to the vacuole[58, 61, 62]. The detection by immunoelectron microscopy of LHCP II within the Golgi apparatus is therefore not surprising when one considers the evolutionary origin of the *Euglena* plastid.

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