Bull. of Nippon Sport Sci. Univ., Vol. 30, No. 1 (2000) 141-152

The Regulation of Chloroplast Synthesis by a Light Mediated Phosphoinositide System

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(Received May 22, 2000; Accepted for publication July 4, 2000)

Four inhibitors of the Phospholipase C-Phosphoinositol [PLC-PIP_2] signaling pathway were used to study the light-mediated synthesis of the chloroplasts of *Euglena gracilis*. Lithium chloride, cadmium chloride, lanthanum chloride and citrate were employed to block steps in the pathway. All inhibitors blocked chlorophyll and carotenoid synthesis is a reversible manner. The kinetics of reversal and potentiation experiments indicated that the inhibition occurred post-transcriptionally/post-translationally. Lithium-inhibited cells showed a block in the transport of LHCP II to the Golgi. Cadmium-inhibited cells showed only partial development and an increase in the appearance of the COS structure that has been implicated in chloroplast protein transport from ER to Golgi. The red-blue light absorbing photomorphogenic system regulating light-induced chloroplast synthesis was sensitive to the inhibition. The data indicate that the initial steps in light-mediated chloroplast synthesis in *Euglena* involve a light induced calcium release from the ER followed by vesicular transport of nuclear-coded chloroplast proteins to the developing chloroplast.

Key words: Phosphoinositide, *Euglena gracilis*, Chloroplast, Proplastid calcium channel

Introduction

Chloroplast synthesis, from its precursor the proplastid, is a light-mediated event in most plants and algae including *Euglena gracilis*. *Euglena gracilis* may be grown in darkness on defined organotrophic medium and the proplastids replicate as cells divide. Upon illumination the proplastids rapidly develop into chloroplasts[1] (Fig. 1a).

While in the bulk of higher plants and most algae nuclear-coded chloroplast proteins are synthesized on free ribosomes and delivered to developing chloroplasts in a post-translational manner, in *Euglena* proteins destined for the chloroplast are processed and sent in a co-translational manner. The chloroplast proteins LHCP II and the small subunit [SSU] of ribulose bisphosphate carboxylase/oxygenase have been localized to the endoplasmic reticulum, the Golgi apparatus and a membranous fraction prior to incorporation into the developing chloroplast.[2-5, 26] *Euglena* is thought to be derived from a secondary symbiotic event wherein a unicellular alga becomes a

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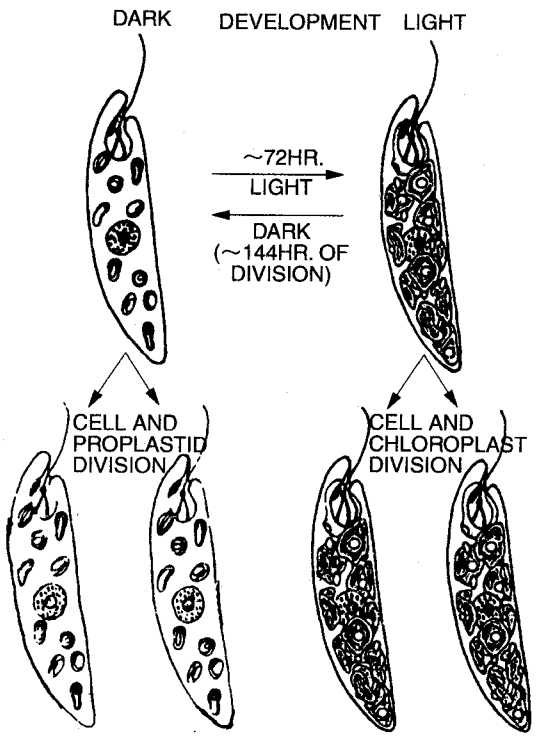

Fig. 1a. Plastid replication and development in *Euglena gracilis* [from ref. 24]

symbiont in a flagellated precursor of the Euglenoids, most likely the phagotrophic diplonemid and kinetoplastid flagellates.[6, 7] Three membranes rather than the two usually seen in higher plants and most green algae surround *Euglena* chloroplasts, and the third membrane is not derived from the endoplasmic reticulum[6] (Fig. 2). While several steps of the protein translocation to *Euglena* chloroplasts have been elucidated,[2-5] little is known about the initial photochemical events, which trigger the synthesis and import of the chloroplast proteins.

We have investigated the initial light-mediated reactions involved in the initiation of chloroplast development in this organism, and present evidence for the involvement of the phosphoinositide transmembrane signaling system. Phosphoinositide signaling [PLC–PIP_2] in plants has been extensively studied. Guard cell aperture control, pulviner movement, calcium channel control in beet root vacuoles, anthocyanin and chlorophyll synthesis in tomato, and swelling of wheat protoplasts have all been shown to be regulated by elements of the PLC–PIP_2 system.[8-12]

The PLC–PIP_2 system involves an external signal, which activates, through G-proteins, phospholipase C [PLC] that hydrolyzes phosphatidylinositol [PIP_2] forming the second messengers inositol triphosphate [IP_3] and membrane-associated diacylglycerol [DAG]. IP_3 opens external or internal calcium channels leading to interaction of calcium with calcium binding proteins and a cascade of cellular events[13] (Fig. 3). IP_3 is dephosphorylated in a series of steps by distinct phosphatases and recycled back into PIP_2 to provide substrate for subsequent activation steps.[14, 15]

We used four inhibitors of the PLC–PIP_2 system to determine if the initial light-mediated steps in chloroplast synthesis are regulated by it. Lithium chloride inactivates the phosphatases involved in recycling IP_3 to IP_2, thus depriving PLC of substrate for subsequent activations.[16] Cadmium chloride and lanthanum chloride block the calcium channels of the endoplasmic reticulum preventing the release of calcium stores.[17] Citrate is a chelator of cytosolic calcium. All classes of inhibitors reversibly blocked chloroplast synthesis. The inhibition by lithium was reversed by washing or by the additional of exogenous inositol. Washing reversed cadmium and lanthanum inhibition. Inhibition by all reagents resulted in partially developed plastids and a block in the transit of chloroplast proteins from the ER to the Golgi apparatus.

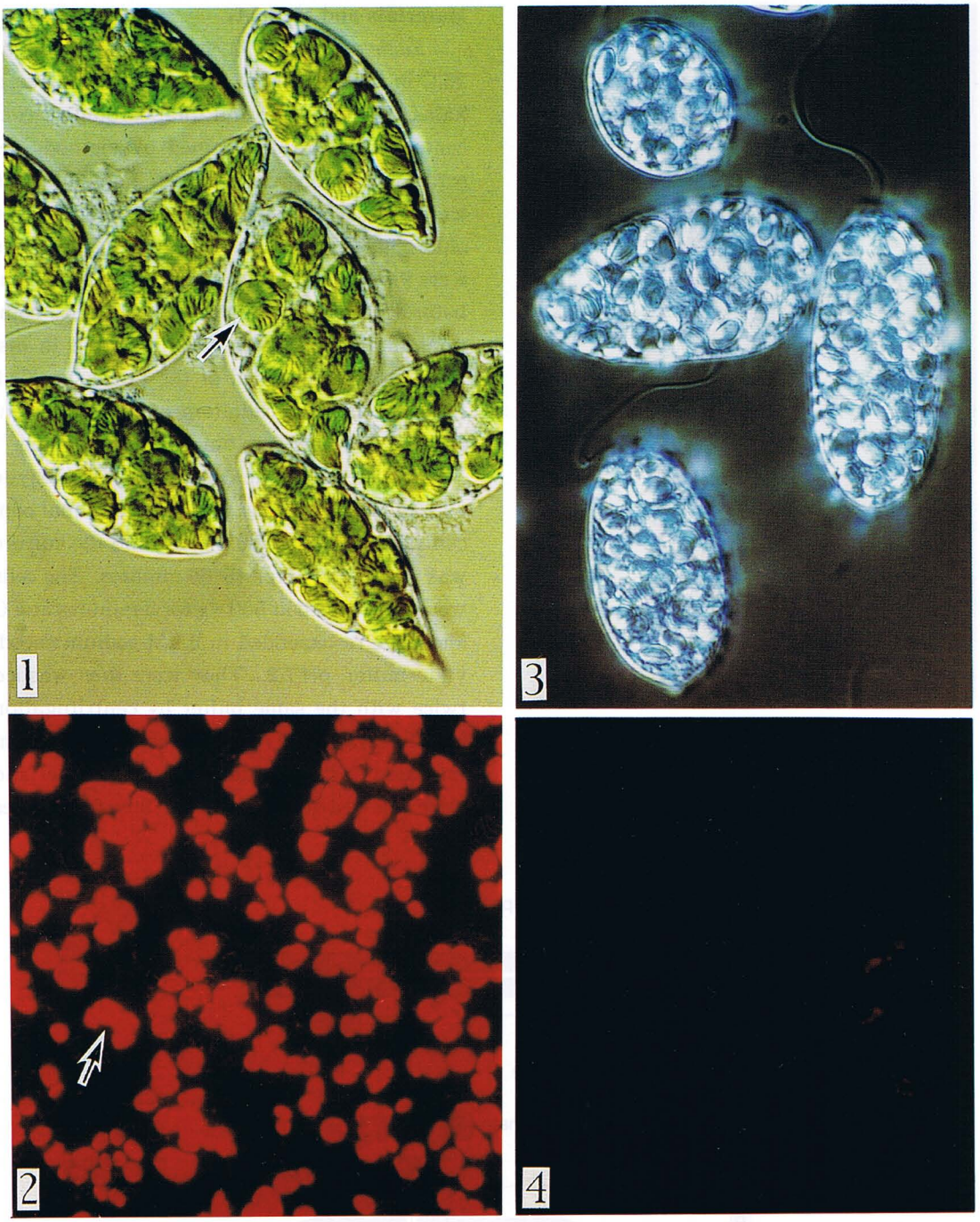

Fig. 1b. Phase and fluorescent micrographs of dark and light grown *Euglena*
1: Phase contrast micrographs of fully green cells. An arrow shows chloroplast. 2: Flourescence micrograph of fully green cells. 3: Phase contrast micrograph of dark grown cells. 4: Flourescence micrograph of dark-grown cells.

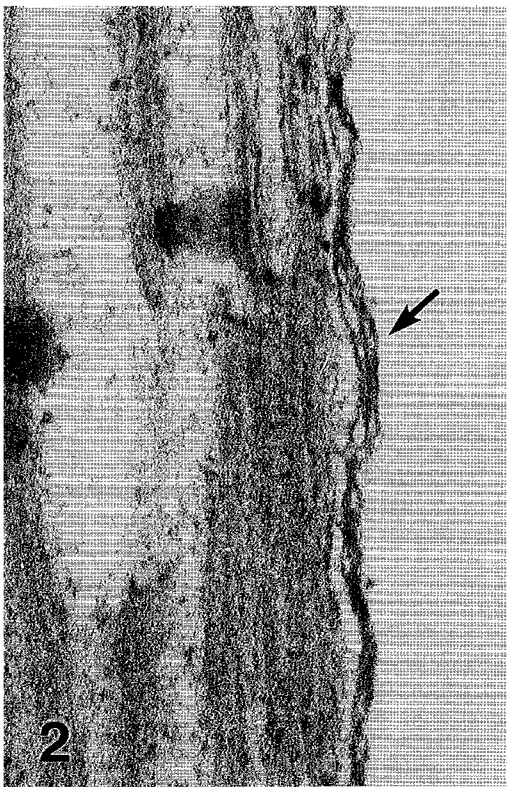

Fig. 2. Electron micrograph of a *Euglena* chloroplast showing three envelope membranes

Materials and Methods

Euglena gracilis, Z strain was grown at 26℃ in the medium of Beale, Foley and Dzelzkalns.[21] Chlorophyll and total carotenoid concentrations were determined by methods described previously.[22] Dark manipulations were carried out using a green safelight. For chloroplast synthesis dark-grown, resting cells were illuminated in a shaking water bath incubator at 26℃ and 6000 lux. Sterile stock solutions of the inhibitors were prepared in distilled water and added at the times specified. Lithium chloride, cadmium chloride and lanthanum chloride were obtained from Sigma.

For electron microscopy, glutaraldehyde was added to a *Euglena* culture to a final concentration of about 2.5% [v/v] and the culture was placed at 4℃ for 45–60 minutes. The cells were centrifuged at 500×*g* for 5 minutes at 4℃ and were resuspended in 0.1 M sodium cacodylate buffer, pH 7.2. They were then washed twice with the same buffer by centrifugation as before and were resuspended in 2% osmium tetroxide in cacodylate buffer or in water and were allowed to incubate for 90–100 minutes

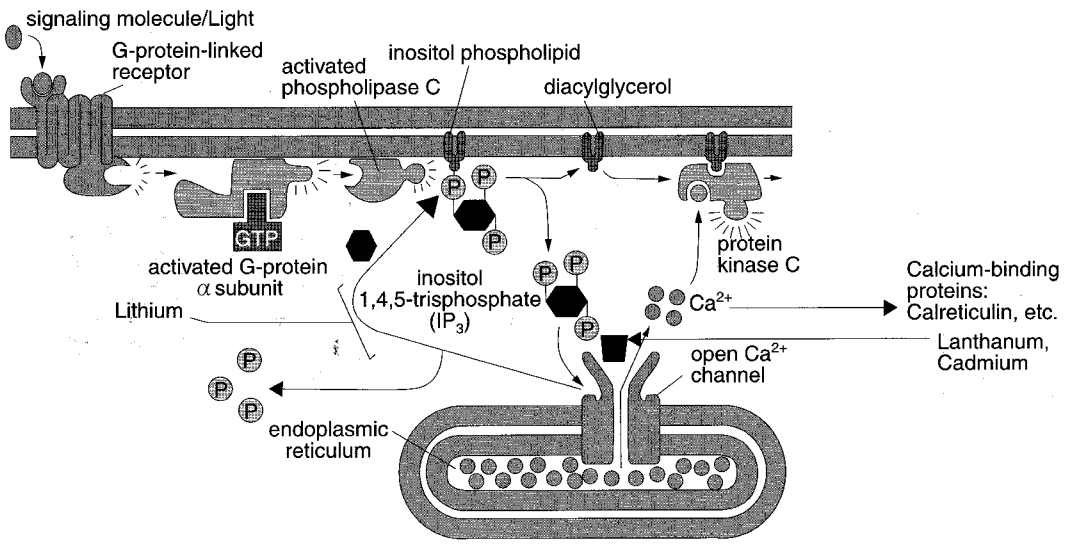

Fig. 3. Diagram of the PLC–PIP_2 transmembrane signaling system

at 4℃. The suspension was then centrifuged, embedded in 2% agar, and dehydrated in a 50 to 90% ethanol series followed by acetone; the material was then embedded in Spurr's resin and serially sectioned on a Reichert microtome. Sections were stained with 2.4% aqueous uranyl acetate overnight at room temperature followed by lead citrate.

Fluorescence microscopy to view chloroplasts in *Euglena* was done using the red fluorescence of chlorophyll as a marker in an Olympus BHS-RFK fluorescence microscope.

For immuno-electron microscopy *Euglena* cells were fixed in glutaraldehyde (final conc. 2%; w/v) in 0.1 M phosphate buffer [pH 7.2] for 60 minutes at 4℃, rinsed in the same buffer and blocked in agar followed by dehydration in ethanol alcohol and then acetone. Samples were embedded in Epon-812 resin [Taab Lab.]. Thin sections were placed on nickel grids. The grids were floated section-side-down on drops of 0.1 M phosphate-buffered saline [PBS] with 0.5% bovine serum albumin for 30 minutes at room temperature, and then incubated with anti-LHCP II antibody in PBS at 37℃ for 10 minutes. The sections were washed twice in PBS, and incubated with protein A-gold in PBS for 20–30 minutes. Sections were subsequently stained with uranyl acetate and viewed in a JEOL 100CX electron microscope at 80 kV.

Results

Chlorophyll and carotenoid synthesis are reversibly inhibited by lithium

Dark-grown, resting cells were exposed to light in varying concentrations of lithium chloride ranging from 14 mM to 72 mM. Figure 4 shows the inhibition of chlorophyll and caro-

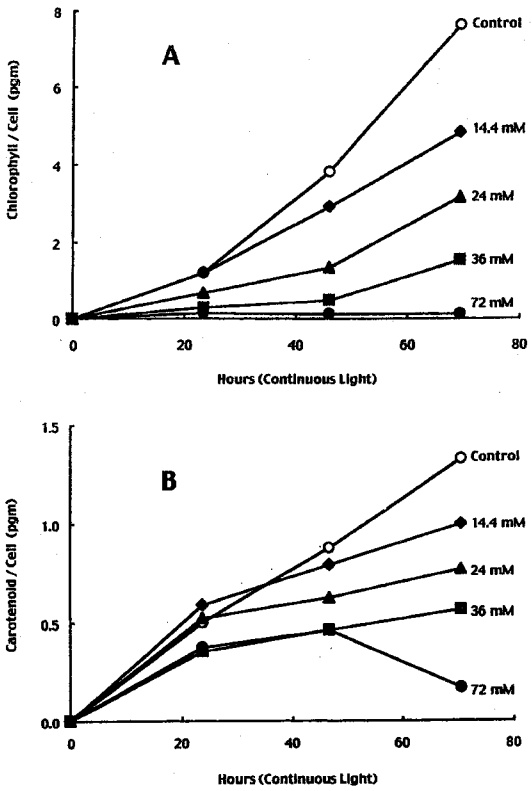

Fig. 4. Chlorophyll [A] and carotenoid [B] inhibition by lithium chloride
Dark-grown resting cells were incubated in varying concentrations of lithium chloride in continuous light. The inhibitory effects of lithium at all concentrations were completely reversible.

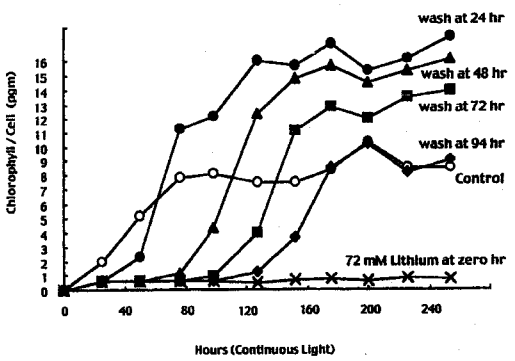

Fig. 5. Reversibility of lithium inhibition of chlorophyll synthesis
Dark-grown resting cells were incubated in the light with 72 mM lithium chloride. At intervals samples were washed twice with sterile medium and allowed to continue chlorophyll synthesis.

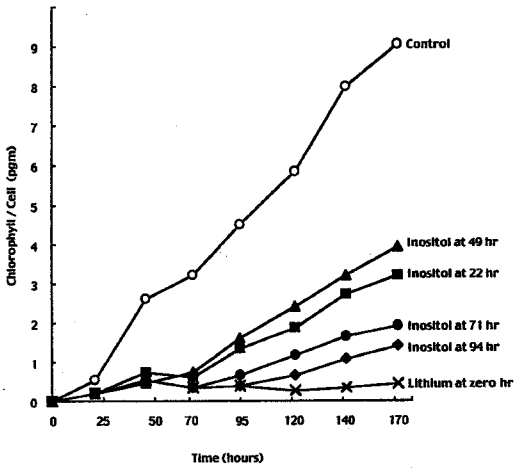

Fig. 6. Reversibility of lithium inhibition by addition of exogenous inositol
Dark-grown resting cells were incubated in the light in 72 mM lithium chloride with varying concentrations of inositol.

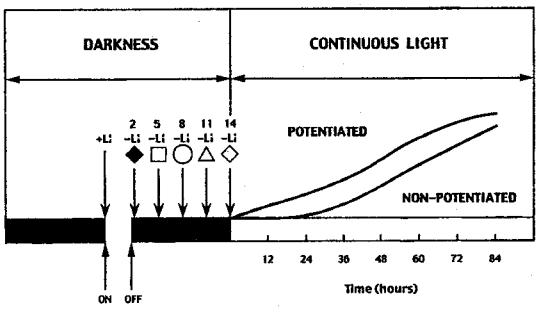

Fig. 7. Diagrammatic representation of potentiation and the times at which lithium chloride was added and removed
Dark-grown cells were incubated in darkness until they reached stationary growth. The cells were exposed to one hour of 6,000 lux and then returned to darkness for twelve hours and then exposed to continuous illumination.

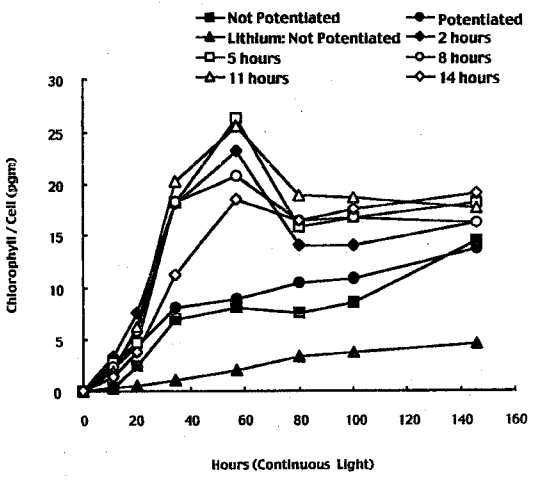

Fig. 8. Effect of lithium chloride on potentiation
Dark-grown, resting cells were incubated in 72 mM lithium chloride at the start of exposure to potentiating light, washed twice at the times indicated in Fig. 7 and then incubated in continuous light.

tenoid by increasing concentrations of lithium chloride. Figure 5 shows the effect of washing the cells after varying times of exposure to 72 mM $LiCl_2$. Cells washed at 48 and 72 hours accumulated more chlorophyll per cell than controls. Washing after 72 hours resulted in control levels of chlorophyll. Carotenoid accumulation was the same as for chlorophyll. Figure 6 shows the partial reversal of 72 mM lithium inhibition by the addition of exogenous inositol. Exogenous inositol even at the highest concentration of lithium was effective in partially reversing the inhibition (Fig. 6).

The accumulation of chlorophyll in cells released from lithium inhibition over controls by at least a factor of two implies the accumulation of chlorophyll precursors during inhibition. To determine if the lithium effect was post-transcriptional or post-translational, we investigated the effect of lithium on potentiation. Potentiation is a returned to darkness, and then exposed to continuous light. Such cells do not show the process wherein dark-grown *Euglena* (with proplastids) are pre-illuminated (potentiated), usual lag in chlorophyll or other chloroplast constituent synthesis due to transcription/translation induced by potentiating light. Transcription/translation induced by the potentiating light continues during the subsequent dark period.[1)]

Figure 7 shows a diagram of potentiation and shows the periods within the potentiating light period where lithium was added and then removed at the onset of continuous light. Figure 8 shows that lithium did not block the potentiated transcription/translation. Also cells exposed to lithium during the potentiation period accumulated about twice the chlorophyll per cell as controls. Therefore it is implied that the lithium inhibition is at a post transcriptional/translational step.

Lithium prevents Golgi accumulation of chloroplast proteins

Cells exposed to lithium chloride failed to direct chloroplast proteins to the Golgi apparatus as seen during normal chloroplast development.[2-5] Figure 14 shows that lithium-inhibited cells accumulated apoLHCP II in the nuclear area. Since the endoplasmic reticulum in *Euglena* is continuous with the nuclear compartment, it can be proposed that inhibition of the PLC–PIP_2 pathway at a post-translational step causes proteins to "back up" on the pathway and accumulate in the endoplasmic reticulum.

In the PLC–PIP_2 system the IP_3 formed binds to endoplasmic reticulum receptors and opens calcium channels. Therefore we utilized blockers of calcium channels to determine if IP_3 formation as suggested by the lithium data is opening calcium channels as part of the regulation of chloroplast synthesis. Chloroplast synthesis in *Euglena* occurs in calcium-free medium and in the presence of EGTA [data not shown].

Calcium channel blockers reversibly inhibit chlorophyll synthesis

The calcium channel blockers, lanthanum and cadmium chloride have been shown to inhibit the PLC–PIP_2 system in other organisms and algae.[15, 17] Figure 9 shows the effect of chlorophyll synthesis of increasing concentrations of these two inhibitors. The effects of both inhibitors could be reversed by washing. Although the data indicate that the release of calcium from internal stores regulates chlorophyll synthesis and chloroplast assembly, the data is equivocal because of the pleiotrophic effects of these inhibitors. It was also noted that cells exposed to 300 mM cadmium chloride and then washed, exhibited a block in cytokinesis but an increased chloroplast division resulting in cells with about 30 small chloroplasts instead of the usual ten. Figure 10b shows such cells. Figure 10a shows that dark-grown cells exposed to cadmium chloride during greening contain, after 48 hours of illumination, an increased number of the COS structures which have been shown to be part of the co-translational pathway followed by

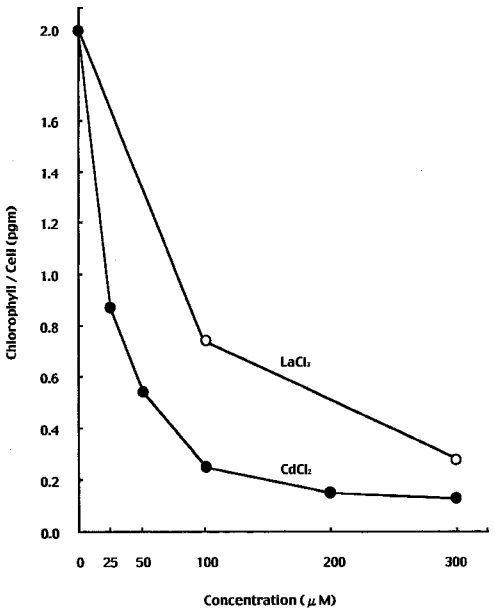

Fig. 9. Dose response curves for inhibition of chlorophyll synthesis by cadmium chloride and lanthanum chloride
Dark-grown, resting cells in varying concentrations of $LaCl_2$ or $CdCl_2$ in sterile, phosphate-free medium were exposed to continuous light. The effects of the various concentrations on chlorophyll synthesis after 48 hours of light are shown.

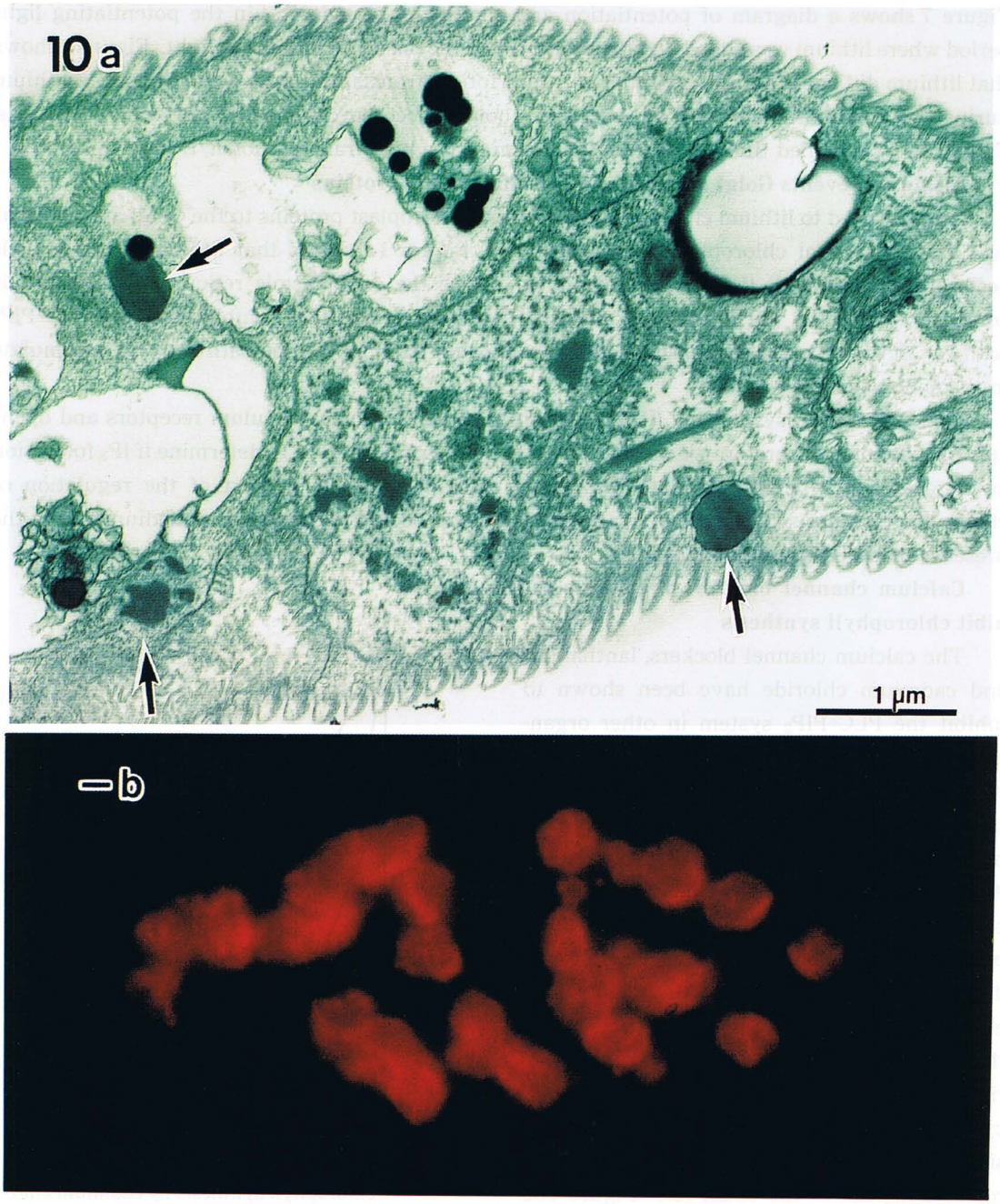

Fig. 10a. Electron micrograph of cadmium inhibited and washed cells
Synchronized cells[25] were incubated in 300 μM cadmium chloride for 5 hours, washed and exposed to continuous light. Chloroplast development is inhibited and an increased amount of COS structures is apparent. The arrows show COS structure.

10b. Flourescence micrograph of cadmium inhibited and washed cells
Compared with control cells synchronized cells[25] were incubated in 300 μM cadmium chloride for 5 hours, washed and exposed to continuous light. Control cells contain about ten chloroplasts while cadmium-treated cells contain about 30 partially developed plastids.

nuclear coded chloroplast proteins in *Euglena*.[5] It is also apparent that the development of the proplastid to the chloroplast has been severely inhibited.

To further assess the role of calcium we utilized the calcium chelator, citrate.

Citrate blocks chlorophyll synthesis

We used sodium citrate as a calcium chelator, because other chelators such as EGTA do not penetrate *Euglena*.

Figure 11 shows the effect of varying concentrations of sodium citrate on chlorophyll synthesis. 400 mM is very inhibitory, 200 mM inhibits about 50% and it is interesting to note that 100 mM is stimulatory and eliminates the lag in chlorophyll synthesis. The inhibition by citrate is reversible [data not shown].

The red-blue photomorphogenic system regulates PLC–PIP_2

Three photomorphogenic systems regulate chloroplast assembly in *Euglena*: A red-blue light absorbing system [probably protochlorophyll], a blue light absorbing system (analogous to the blue-UVA systems in higher plants) and a 600 nm absorbing system.[1, 22] The blue light and 600 nm systems may be assayed in a background of saturating red light. In saturating red light, the addition of blue or 600 nm light will enhance chlorophyll and carotenoid synthesis. Figures 12 and 13 show that in the lithium inhibits chlorophyll synthesis in saturating red light, but that the enhancement by either blue or 600 nm light is still apparent, in the presence of the inhibitor. This indicates that the action[s] of the red-blue system is lithium sensitive and that the red-blue photomorphogenic system is activating the PLC–PIP_2 signaling system.

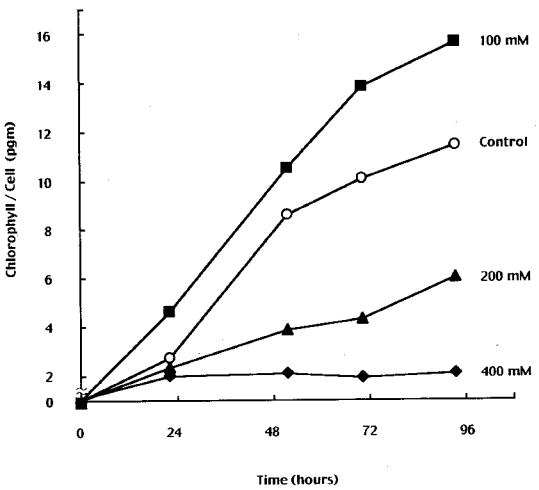

Fig. 11. Inhibition of chlorophyll synthesis by sodium citrate
Dark-grown, resting cells were exposed to varying concentrations of sodium citrate, and the effect on chlorophyll synthesis was determined. 200 mM and 400 mM were inhibitory while 100 mM was stimulatory.

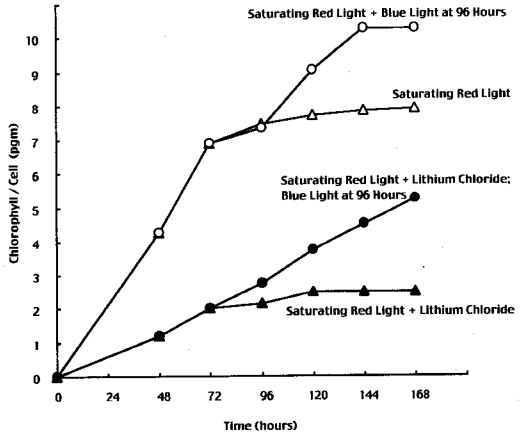

Fig. 12. Effect of lithium chloride on the blue photomorphogenic system
Dark-grown, resting cells were incubated in 72 mM lithium chloride in red light until saturation was reached, and then a second beam of blue light was added to the red light. Cells incubated in saturating red light were inhibited by lithium chloride, but still showed the blue light enhancement indicating that the blue ligth photomorphogenic system is not sensitive to lithium inhibition.

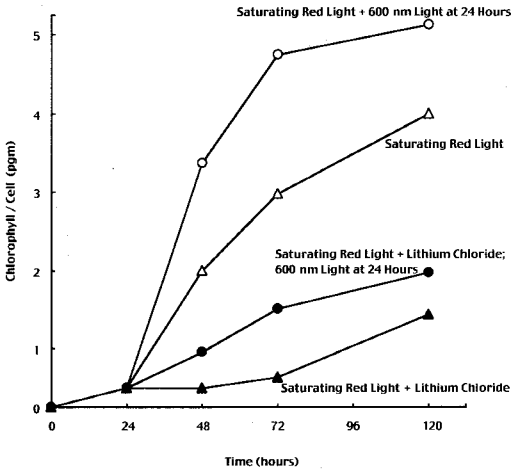

Fig. 13. Effect of lithium chloride on the 600 nm photomorphogenic system
Cells were incubated as in Fig. 12, but the additional light added at saturation was 600 nm. Lithium-incubated cells show enhancement by 600 nm light indicating that the 600 nm system is not sensitive to lithium chloride inhibition.

Discussion

The data presented above imply that a PLC–PIP_2 signaling system, activated by the red-blue light absorbing system, initiates the assembly of the chloroplast in *Euglena gracilis*. Further, the potentiation experiments and the excess chlorophyll produced upon removal of lithium indicate that the PLC–PIP_2 system is operating post-transcriptionally/translationally. The excess chlorophyll seen upon removal of lithium could be interpreted as the "piling up" of chloroplast precursors en route to the developing plastid. The effects of calcium channel blockers and calcium chelators suggest that calcium is involved in these initial events in chloroplast assembly. The chlorophyll-binding protein, LHCP II and the small subunit [SSU] of the stromal ribulose bisphosphate carboxylase/oxygenase are coded by the nucleus and reach the developing chloroplast by a co-transcriptional mechanism.[2] Both proteins are synthesized as membrane-bound polyproteins, traverse the endoplasmic reticulum and Golgi apparatus, and are delivered to the chloroplast in a sodium carbonate insensitive (vesicular?) fraction.[2–4] Calcium has been implicated in post-Golgi vesicular traffic, and it is tempting to speculate that this role is occurring in *Euglena*. There are problems with this speculation however.

The only calcium-binding protein found to date in *Euglena* is calreticulin and it is restricted to the endoplasmic reticulum (ER).[23] ER-localized calreticulin acts as a chaperone for proteins traveling the co-transcriptional route. It is possible that calreticulin acts in the ER of *Euglena* in a dark-repressor fashion. SSU proteins are synthesized during dark growth in small amounts and found in the proplastid. It is possible that the calcium–calreticulin–polyprotein complex in the ER acts to prevent vesicular transport to the proplastid in the dark thus acting as a repressor of chloroplast synthesis, and a feedback inhibitor of further chloroplast protein synthesis. Western blot analysis of lithium inhibited cells show a decrease of LHCP II and SSU [Schwartzbach: personal communication], and immuno-electron microscopic analysis shows the proteins "backing up" into the nucleus (Fig. 14). In *Euglena* the ER and nuclear compartment form a continuous network. In this proposed mechanism, light would open the ER calcium channels; the calcium–calreticulin–polyprotein complex would be altered by calcium export to the cytosol and vesicular transport would proceed. This does not preclude a role for the exported calcium in vesicular transport or other events and indeed, given the parsimonious nature of cellular regulation, this is likely. For instance, LHCP II is not synthesized in the dark while a small amount of SSU is found in dark-grown cells.[2,5] Cytosolic calcium might be involved in the attachment of ribosomes with LHCP II m-RNA to the ER or in the insertion of the protein during co-translational synthesis.

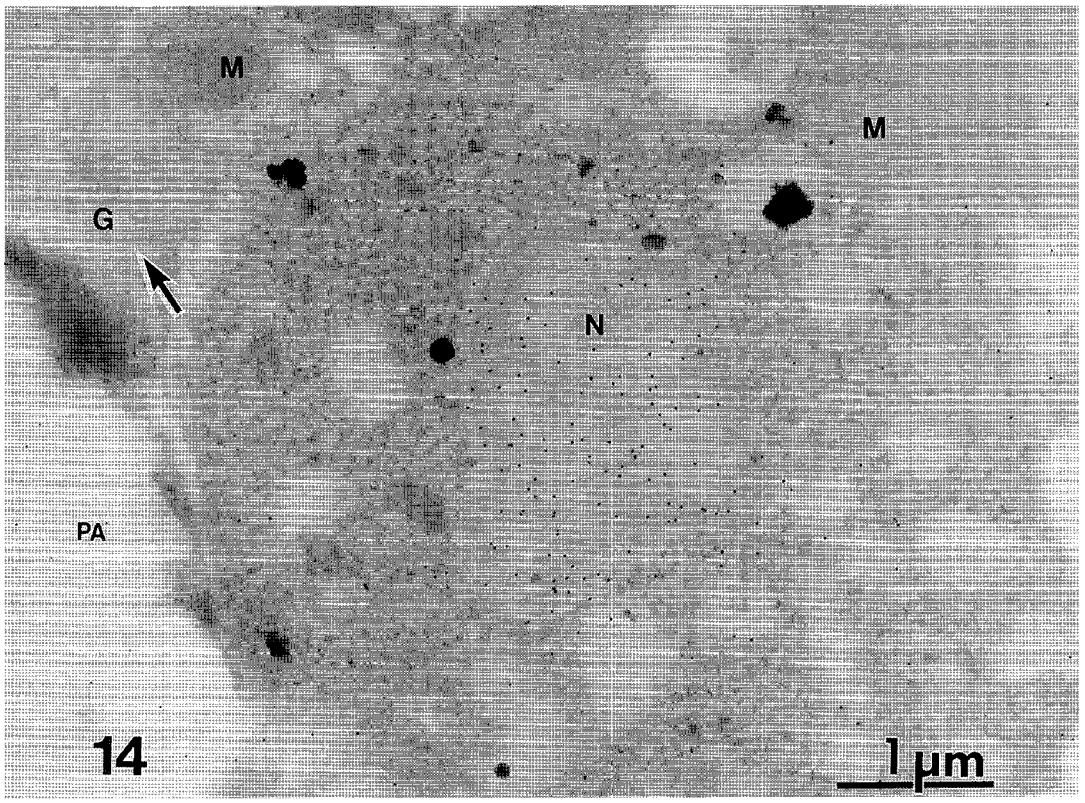

Fig. 14. Immuno-electron micrograph of lithium inhibited cells
Cells were incubated as in Fig. 5 and prepared for immunocytochemical localization. Inhibited cells show LHCP II antibody over the nuclear area; control cells show antibody accumulated in the Golgi.

Conclusions

1. Light-mediated chloroplast synthesis in *Euglena* is regulated by a PLC–PIP_2 system.
2. Inhibitors of the PLC–PIP_2 system, lithium chloride and cadmium chloride, reversibly block chloroplast development at a post-translational step.
3. The initial steps in light-mediated chloroplast involve a light-mediated calcium release from the ER followed by vesicular transport of nuclear-coded chloroplast proteins to the developing chloroplast.

Acknowledgments

This work was aided by grants from the Ministry of Education, Science, Sports and culture in Japan (No. 09640795) and the Kazato Research Foundation (2000).

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