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A 36 Kilodalton Limiting-CO_2 Induced Polypeptide of *Chlamydomonas* Is Distinct from the 37 Kilodalton Periplasmic Carbonic Anhydrase[1]

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ABSTRACT

***Chlamydomonas reinhardtii* possesses a CO_2-concentrating mechanism, induced by limiting CO_2, which involves active transport and accumulation of inorganic carbon within the cell. Synthesis of several proteins is induced by limiting CO_2, but, of those, only periplasmic carbonic anhydrase has an identified function in the system. No proteins involved in active transport have yet been identified, but induced, membrane-associated polypeptides, such as the 36 kilodalton polypeptide focused on in this paper, would seem to be candidates for such involvement. The 36 kilodalton polypeptide was shown to be synthesized *de novo* upon transfer of cells to limiting CO_2. It was purified using SDS-PAGE and used to produce polyclonal antibodies. Antibodies were used to confirm the air-specific nature of the polypeptide, its strict association with membrane fractions, and the time course of its induction. Using the antibodies, a single, 36 kilodalton polypeptide was found to be specifically immunoprecipitated from *in vitro* translation products of poly(A^+) RNA from cells only after exposure to limiting CO_2. The absence of translatable mRNA for this polypeptide in CO_2-enriched cells indicated that regulation occurs at the level of message abundance. The antibodies were also used to demonstrate the distinction between the limiting-CO_2 induced 36 kilodalton polypeptide and the similarly sized, limiting-CO_2 induced periplasmic carbonic anhydrase.**

Chlamydomonas reinhardtii, as well as several other green microalgae and cyanobacteria, exhibit a CO_2-concentrating system induced by low external levels of CO_2. This system (reviewed in 1, 13, and 15), which gives them a high apparent affinity for CO_2, results from the active transport of inorganic carbon (C_i)[2]. Active transport of C_i raises the intracellular CO_2 level allowing photosynthesis to be saturated at a lower external CO_2 concentration and raising the CO_2:O_2 ratio, thereby inhibiting the oxygenase activity of rubisco. These cells have a dramatically reduced rate of photorespiration and show little, if any, O_2 inhibition of photosynthesis. The CO_2-concentrating system is inducible; cells grown at enriched (5%) CO_2 levels (CO_2-enriched cells) do not exhibit CO_2 concentrating activity. When cells are grown in the air (0.03% CO_2; air-adapted cells), their apparent affinity for CO_2 is much higher than that of CO_2-enriched cells.

Components of the CO_2-concentrating mechanism include a mechanism for active transport of C_i, an internal carbonic anhydrase, and a periplasmic carbonic anhydrase (1, 15). Induction of the CO_2-concentrating mechanism and periplasmic carbonic anhydrase occur in response to low external C_i. Lack of induction in the presence of CHI indicates that *de novo* protein synthesis is essential (5, 12, 17). Translation of one or more proteins involved in the system must be initiated or upregulated.

The best-characterized protein for which specific involvement in the system and a function have been identified is the periplasmic carbonic anhydrase. Coleman *et al.* (6) identified a soluble 37 kD polypeptide as the periplasmic carbonic anhydrase in *Chlamydomonas*. The polypeptide appears following transfer of CO_2-enriched cells to air conditions. It is excreted into the medium by the CW15 mutant which lacks a normal cell wall.

The synthesis of other polypeptides has been correlated with the induction of the CO_2-concentrating mechanism, but so far none have been assigned a specific function or location. It is possible that one or more of these proteins could be involved with the transport mechanism, since neither the nature of the transporter nor its location has been determined. Along with noting a transitory decline in the synthesis of certain polypeptides such as the rubisco subunits during the induction phase, Bailly and Coleman (2) reported the transitory synthesis of 49 and 52 kD soluble polypeptides and the prolonged synthesis of a 20 kD soluble polypeptide in addition to the 37 kD periplasmic carbonic anhydrase. Manuel and Moroney (10) have reported polypeptides of similar molecular mass (20, 37, 44, and 46 kD) which they found to be heavily labeled during the induction phase in crude cell homogenates. Spalding and Jeffrey (16) have identified four membrane-associated polypeptides (molecular masses 19, 21, 35, and 36 kD) and two soluble polypeptides (molecular mass 45–50 kD) which appear or increase in abundance during induction of the CO_2-concentrating mechanism. Although the appearance of the 19 and 35 kD polypeptides was somewhat variable and lagged behind the increase in photosynthetic rate, the appearance of the 21 and 36 kD polypeptides seemed well coordinated with the induction of the CO_2-concentrating mechanism (16). The 36 and 21 kD polypeptides were also reported to be present only in trace amounts in purified thylakoid membranes (16), indicating they are associated with mem-

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[2] Abbreviations: C_i, inorganic carbon; CAP, chloramphenicol; CHI, cycloheximide; ACA, amino caproic acid; BAM, benzamidine; rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

branes other than the thylakoids. Since cell wall-less cells were used, there should have been no periplasmic carbonic anhydrase present, in which case the 35 and 36 kD polypeptides should be different from the previously identified 37 kD periplasmic carbonic anhydrase.

The 36 kD limiting-CO_2 induced polypeptide, LIP-36, was isolated and used to produce polyclonal antibodies. The antibodies confirm the correlation of the appearance of LIP-36 with the CO_2-concentrating system. They also distinguish LIP-36 from the 37 kD periplasmic carbonic anhydrase on the basis of sub-cellular location, primary translation product size and immunological reactivity.

MATERIALS AND METHODS

Algal Strain and Culture Conditions

Chlamydomonas reinhardtii strain CW15 mt^+, a cell wall-less mutant (Dr. R. Togasaki, Indiana University), was grown in a minimal salts medium consisting of 143 mg/L K_2HPO_4, 73 mg/L KH_2PO_4, 400 mg/L NH_4NO_3, 100 mg/L $MgSO_4 \cdot 7 H_2O$, 50 mg/L $CaCl_2 \cdot 2 H_2O$, 1 mL/L trace elements stock (18), and 10 mL/L 2.0 M Mops titrated with Tris base to pH 7.1 for air medium and to pH 7.6 for CO_2 medium. The pH of cultures during growth was 6.9 to 7.1. Liquid cultures were grown at room temperature on a gyratory shaker (175 rpm) under constant light (100 μ E m^{-2} s^{-1}). CO_2-enriched cells were aerated with 5% CO_2 in air. For timed inductions, cells were grown under CO_2-enriched conditions, collected by centrifugation, resuspended, and grown under air-adapted conditions (no aeration). To avoid possible message induction during centrifugation and resuspension, cells grown with CO_2 enrichment were either maintained at 5% CO_2 (CO_2-enriched) or flushed with air for 30 min and incubated without aeration for an additional 3½ h (4 h air-adapted) for *in vitro* translation experiments. For translation inhibition experiments, cells were grown under CO_2-enriched conditions, collected by centrifugation, and resuspended in sulfate-free air-medium. Inhibitors were added (0.2 mg/mL CAP; 0.01 mg/mL CHI) and cultures kept in the dark for 10 min, then grown under air-adapted conditions for 4 h. [^{35}S]Sulfate (100 μCi/100 mL) was added for the last 3 h.

Cell Fractionation

Cells were harvested by centrifugation (2000 rpm, Sorvall GSA rotor, 5 min), washed with buffer A (10 mM Tris-HCl, 10 mM EDTA, 5 mM ACA, 2 mM BAM [pH 7.5]), recentrifuged (2000 rpm, Sorvall SS-34 rotor, 5 min), and resuspended in 10 mL buffer A with 0.25 mL PMSF (40 mM in isopropanol) added. Whole cell samples were taken at this point. The remaining cells were ruptured using a nitrogen pressure bomb (Parr model 4639; 1000 psi). The resulting suspension was centrifuged (1000 rpm, IEC clinical centrifuge, 5 min) to remove unbroken cells. The supernatant was then centrifuged (19,000 rpm, Sorvall SS-34 rotor, 30 min) to pellet the total membrane fraction. Alternatively, the supernatant was centrifuged at 5000 rpm (30 min) then 19,000 rpm (30 min) to separate high-speed and low-speed membranes (16). The membrane pellets were resuspended in buffer A, and the supernatant was saved as the soluble fraction. Extracellular protein was collected by ammonium sulfate precipitation (70% saturated) of the growth medium from air-adapted CW15 cells. The precipitated protein was resolubilized in buffer A and desalted by repeated centrifugal concentration in an Amicon Centricon 10 concentrator. The above steps were all done at 4°C.

Gel Electrophoresis

Samples were run on 10–18% gradient polyacrylamide gels (10–18% acrylamide, 0.8:30 bis-acrylamide:acrylamide) using the Laemmli buffer system (8), except that no SDS was added to the lower reservoir buffer. Gels were stained with silver (7) or Coomassie brilliant blue (3), and labeled polypeptides were detected by fluorography (14) or autoradiography. Molecular mass markers used for stained gels were phosphorylase *b* (97 kD), BSA (68 kD), glutamate dehydrogenase (53 kD), ovalbumin (43 kD), glyceraldehyde-3-P dehydrogenase (36 kD), bovine carbonic anhydrase (29 kD), soybean trypsin inhibitor (20 kD), and Cyt *c* (12 kD) obtained from Sigma. For fluorography, the ^{14}C-labeled molecular mass marker kit obtained from Sigma contained BSA (68 kD), ovalbumin (43 kD), glyceraldehyde-3-P dehydrogenase (36 kD), bovine carbonic anhydrase (29 kD), trypsin inhibitor (20 kD), and α-ketalbumin (14 kD).

Protein Isolation

The 36 kD limiting-CO_2 induced polypeptide (LIP-36) was isolated from the total membrane fraction (see above) of air-adapted cells by SDS-PAGE. Gels were stained with Coomassie brilliant blue and partially destained. The 36 kD band, identified by comparison with CO_2-enriched membranes, was cut out and homogenized in buffer A, then gently agitated overnight at 4°C to allow diffusion of the protein into the buffer. The polyacrylamide was removed by centrifugation and the resulting supernatant concentrated at 4°C with an Amicon Centricon 10 microconcentrator. The protein was electrophoresed a second time for additional purification and recovered in the same manner.

Antibody Production and Immunodetection

Polyclonal antibodies were produced in a female New Zealand white rabbit using a technique developed for small doses of immunogen (20). Protein samples were separated by SDS-PAGE as previously described. Protein was electrophoretically transferred from the gel to nitrocellulose (19). The primary antibody signal was amplified using biotinylated anti-rabbit IgG as a secondary antibody and detected using streptavidin-linked horseradish peroxidase.

RNA Isolation, *in Vitro* Translation, and Immunoprecipitation

Cells were harvested by centrifugation (5000 rpm, Sorvall GSA rotor, 5 min), immediately resuspended in lysis buffer (15 mM EDTA, 1% [w/v] SDS, 200 mM NaCl, 5 mM DTT, 0.2 mg/ml proteinase K, 50 mM Tris-HCl [pH 8.0]), extracted

twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform, then precipitated with ethanol (67% v/v). Total RNA was precipitated with 2 M LiCl overnight, followed by two additional ethanol precipitations. Poly(A^+) RNA was purified by poly(U) Sephadex column chromatography (9).

In vitro translation of poly(A^+) RNA was carried out using L-[^{35}S]methionine (New England Nuclear) with nuclease treated, rabbit reticulocyte lysate (Promega) according to the manufacturers instructions. [^{35}S]Methionine incorporation was routinely monitored as trichloroacetic acid precipitable label incorporation.

Immunoprecipitations were carried out using the method described by Colbert *et al.* (4), except protease inhibitors (5 mM ACA, 1 mM BAM, 10 mM iodoacetamide, and 1 mM PMSF) were included in the immunoprecipitation solution, protein A Sepharose was used instead of *Staphylococcus aureus* and 0.05% (w/v) sodium azide was included in all stock solutions. Each translation supernatant was immunoprecipitated with preimmune serum (20 μL) prior to final immunoprecipitation with LIP-36 antiserum (20 μL). Total *in vitro* translation products and immunoprecipitated translation products were analyzed by SDS-PAGE as described above.

RESULTS AND DISCUSSION

Although the existence of a CO_2-concentrating mechanism in *Chlamydomonas* has been demonstrated, no components other than the periplasmic carbonic anhydrase have been identified. Proteins, such as LIP-36, whose synthesis is correlated with the induction of the CO_2-concentrating mechanism could be components of the CO_2-concentrating system or involved in its regulation. Characterization of such proteins may help determine the nature of the mechanism and its regulation. LIP-36 was selected for this work based on the time course of its appearance and its association with nonthylakoid membranes.

To determine whether LIP-36 is encoded by a nuclear gene or a plastid gene, proteins synthesized during induction of the CO_2-concentrating mechanism in the presence or absence of CHI and CAP were labeled and compared. CHI, which inhibits translation on cytosolic ribosomes, inhibited synthesis of LIP-36, as well as the 21 kD membrane-associated polypeptide, the two 45 to 50 kD soluble polypeptides and a 45 kD apparently air-induced polypeptide in the membrane fraction (Fig. 1, lanes 4 and 8). The 45 kD polypeptide in the membrane fraction was not routinely observed to be induced at limiting CO_2. These proteins were synthesized in the presence of CAP, which inhibits translation on plastid ribosomes (Fig. 1, lanes 3 and 7). These results indicate that the induced proteins are probably encoded by nuclear genes. The absence of the proteins in the CHI-treated lanes of the stained gel indicates that the proteins are synthesized *de novo* rather than from constitutive precursors.

To further characterize LIP-36, the protein was purified (Fig. 2, lane 1) as described above and used to produce polyclonal antibodies. On Western blots preimmune serum exhibited no detectable reactivity with membrane proteins of either cell type (data not shown), whereas immune serum specifically recognized a 36 kD polypeptide in the membrane

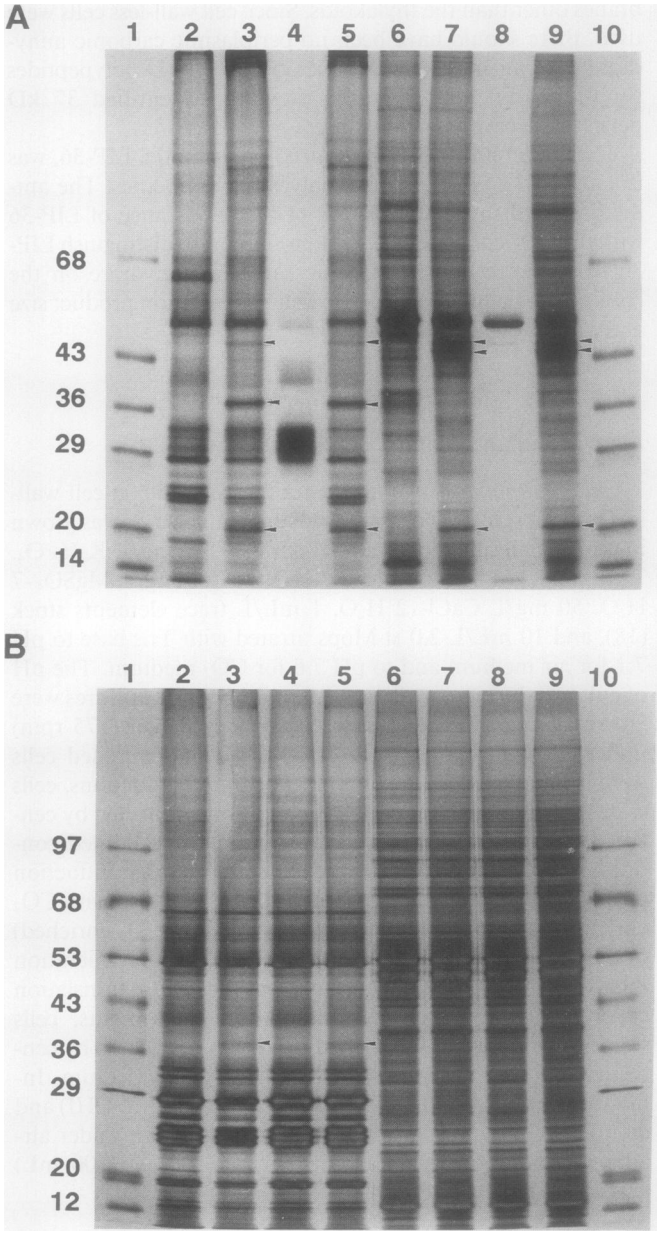

Figure 1. Fluorograph (A) and silver-stained (B) SDS-PAGE gel of membrane fractions (lanes 2–5) and soluble proteins (lanes 6–9) from *C. reinhardtii*. Cells were CO_2-enriched (lanes 2 and 6), CO_2-enriched transferred to air for 4 h (lanes 3 and 7), and CO_2-enriched transferred to air for 4 h in the presence of CHI (lanes 4 and 8) or CAP (lanes 5 and 9). Lanes 1 and 10 contain molecular size markers. Arrows indicate polypeptides apparently induced by air.

fraction only of air-adapted cells (Fig. 3, lanes 10 and 13). It is somewhat surprising that the antibodies are so specific, since purification consisted only of two sequential SDS-PAGE purifications beginning with a crude membrane fraction (*cf.* Fig. 3, lane 3). It was expected that adsorption of the antiserum against membrane proteins from CO_2-enriched cells might be required to produce a specific antibody preparation. As can be seen (Fig. 3), such steps proved unnecessary.

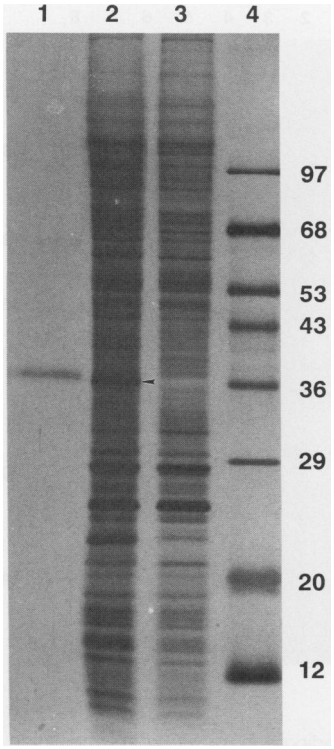

Figure 2. Silver-stained SDS-PAGE gel of purified LIP-36 (lane 1) and high-speed membrane fractions from air-adapted cells (lane 2) and CO_2-enriched cells (lane 3) of *C. reinhardtii*. Lane 4 contains molecular size markers. Arrow indicates LIP-36.

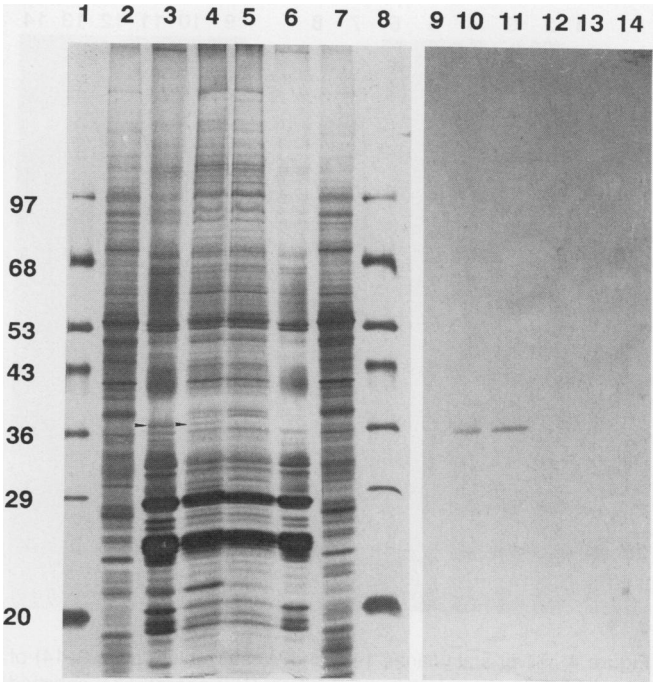

Figure 3. Silver-stain (lanes 1–8) and Western blot (lanes 9–14) of SDS-PAGE gel of whole cell samples (lanes 4, 5, 11, and 12), membrane fractions (lanes 3, 6, 10, and 13), and soluble proteins (lanes 2, 7, 9, and 14) from air-adapted (lanes 2–4 and 9–11) and CO_2-enriched (lanes 5–7 and 12–14) cells of *C. reinhardtii*. Antiserum was diluted 1:100. Lanes 1 and 8 contain molecular size markers. Arrows indicate LIP-36.

In a Western blot comparing fractionated cells and whole cells of CO_2-enriched and air-adapted *Chlamydomonas* no detectable LIP-36 was observed in the soluble fraction of air-adapted cells (Fig. 3, lane 9). This observation confirms the previous identification, by staining and fluorography, of LIP-36 as a membrane-associated polypeptide (16) in experiments where its simultaneous presence in the soluble protein fraction might not have been detected. There was also no reactivity detected in either fraction or in total cell protein of the CO_2-enriched cells (Fig. 3, lanes 12–14). The polypeptide had previously been identified as air-specific solely on the basis of its apparent molecular mass, so its presence in CO_2-enriched cells at low abundance or at a different apparent molecular mass could not be ruled out until now. These results are consistent with those of the translation inhibitor experiment, which indicate that LIP-36 is synthesized *de novo* in air-adapted cells rather than being modified from a preexisting polypeptide.

Samples taken from air-adapted and CO_2-enriched cell cultures prior to cell fractionation showed that LIP-36 in the whole cell sample exhibited antibody reactivity similar to that observed for membrane fractions, with no additional nonspecific binding detected (Fig. 3, lane 11). This observation allowed the use of whole cell samples in further experiments.

The results of a time course of LIP-36 induction following transfer from CO_2-enriched to air conditions are shown in Figure 4. Although LIP-36 was not clearly visible in whole cell samples until 8 h after transfer in the silver-stained gel (earlier detection has been reported in membrane fractions by Spalding and Jeffrey 16), the antibodies were able to detect low levels present at 2 h, with substantial levels reached by 8 h (Fig. 4). These results are similar to the time course of induction of both increased photosynthetic rates and C_i accumulation (16), which is consistent with the possibility that LIP-36 is involved with C_i transport.

LIP-36 antiserum was used to immunoprecipitate the LIP-36 primary translation product from *in vitro* translation (Fig. 5). Although an apparent air-specific band at 36 kD can be seen in the autoradiograph of total translation products (Fig. 5, lanes 1 and 2), this band was not routinely observed so may not be related to LIP-36. The antiserum precipitated a single translation product of approximately 36 kD from *in vitro* translation of poly(A^+) RNA of 4 h air-adapted but not of CO_2-enriched cells (Fig. 5, lanes 3 and 4). Prolonged fluorographic exposure of the immunoprecipitated *in vitro* translation products demonstrated no detectable LIP-36 in the translation products from poly(A^+) RNA of CO_2-enriched cells (Fig. 5, lanes 6 and 7). These observations indicate that regulation of LIP-36 occurs through regulation of mRNA abundance, possibly at the level of transcript initiation, processing or stability. Immunoprecipitation of LIP-36 from *in vitro* translation products of poly(A^+) RNA is also consistent with LIP-36 being translated on cytosolic ribosomes, as suggested by the translation inhibitor results.

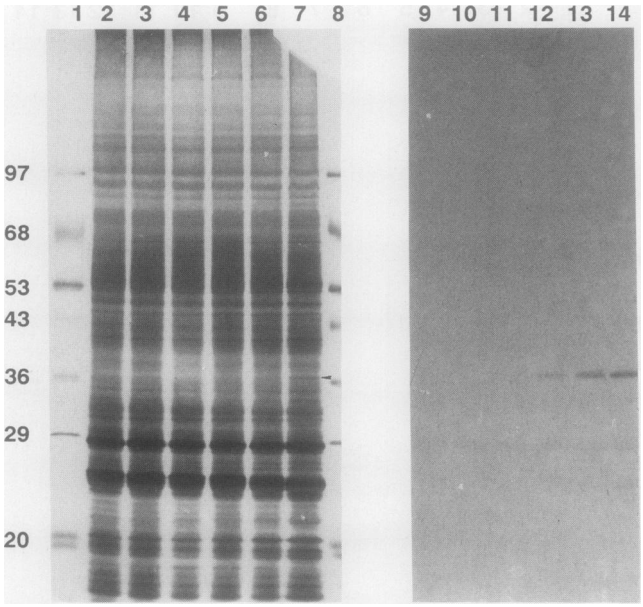

Figure 4. Silver-stain (lanes 1–8) and Western blot (lanes 9–14) of SDS-PAGE gel of whole cell samples from *C. reinhardtii* CO_2-enriched cells (lanes 2 and 9) and CO_2-enriched cells transferred to air for 1 h (lanes 3 and 10), 2 h (lanes 4 and 11), 4 h (lanes 5 and 12), 8 h (lanes 6 and 13), and 24 h (lanes 7 and 14). Antiserum was diluted 1:100. Lanes 1 and 8 contain molecular size markers. Arrow indicates LIP-36.

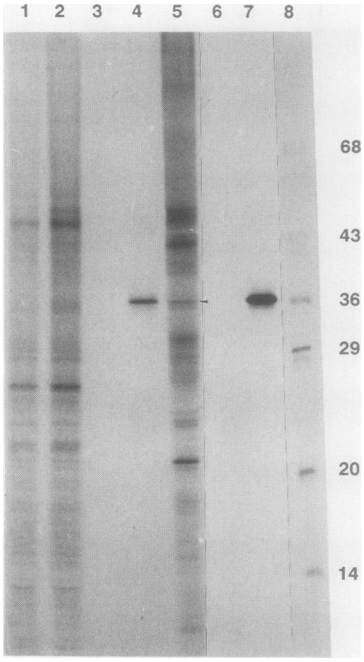

Figure 5. Autoradiograph of *in vitro* translation products from poly(A^+) RNA (lanes 1 and 2), polypeptides immunoprecipitated from *in vitro* translation products with LIP-36 antiserum (lanes 3 and 4), *in vivo* translation products (lane 5), and fluorograph of polypeptides immunoprecipitated from *in vitro* translation products with LIP-36 antiserum (lanes 6 and 7), all separated by SDS-PAGE. Lanes 1, 3, and 6 represent CO_2-enriched cells, and lanes 2, 4, 5, and 7 represent 4 h air-adapted cells of *C. reinhardtii*. Lane 8 contains molecular size markers. Arrow indicates LIP-36.

The immunoprecipitated translation product was of the same apparent molecular mass as LIP-36 (36 kD) or only slightly larger (Fig. 5, lanes 4 and 5). From these results it is not possible to determine definitively whether LIP-36 is synthesized as a larger precursor. However, if it is proteolytically processed following translation, the cleaved presequence must be fairly short.

LIP-36 is very close in size to the 37 kD periplasmic carbonic anhydrase previously identified in *Chlamydomonas* (6), which also is induced by limiting CO_2. It therefore seemed possible that LIP-36 was a membrane-associated form of the periplasmic carbonic anhydrase or that it was simply a contamination of the membrane fraction by this very abundant protein. Although LIP-36 appeared to have a slightly smaller apparent molecular mass (Fig. 6, lanes 3 and 4), further evidence was desired to determine its uniqueness. A cross-reactivity experiment was performed using extracellular protein as a crude periplasmic carbonic anhydrase sample and periplasmic carbonic anhydrase antiserum obtained from Dr. H. David Husic, (Lafayette College, Easton, PA). While there seemed to be significant binding of the periplasmic carbonic anhydrase antiserum to various proteins in the membrane fraction (Fig. 6, lanes 5 and 6), and it clearly recognized the periplasmic carbonic anhydrase (Fig. 6, lane 7), there was no detectable binding to LIP-36 (Fig. 6, lane 6). Conversely, the LIP-36 antiserum did not bind at all to the periplasmic carbonic anhydrase (Fig. 6, lane 10). It is concluded, therefore, that the two proteins are distinct. LIP-36 has not been reported by other groups, probably because its presence is masked by or confused with the periplasmic carbonic anhydrase when they are not separated by fractionation.

In summary, it has been shown that a 36 kD membrane-associated polypeptide, LIP-36, is present in air-adapted, but not CO_2-enriched cells. Purification of LIP-36 and production of polyclonal antibodies allowed for an immunological confirmation of the air-specific nature of this polypeptide, previous identification of which was based on staining and fluorography (16). It was also demonstrated that the polypeptide is synthesized *de novo* on cytosolic ribosomes upon induction of the CO_2-concentrating system, and an induction time course showed that synthesis of the polypeptide is induced along approximately the same time course as the CO_2-concentrating system.

Immunoprecipitation of the LIP-36 primary translation product from *in vitro* translation demonstrated that translatable mRNA for LIP-36 was only present in cells exposed to limiting CO_2, indicating regulation at some point preceding translation. Immunoprecipitation of the primary translation product also demonstrated that LIP-36 is either translated at its mature size or contains only a relatively short cleaved presequence.

It has further been shown that LIP-36 is distinct from the previously identified 37 kD periplasmic carbonic anhydrase. The apparent molecular mass of LIP-36 is slightly lower, and

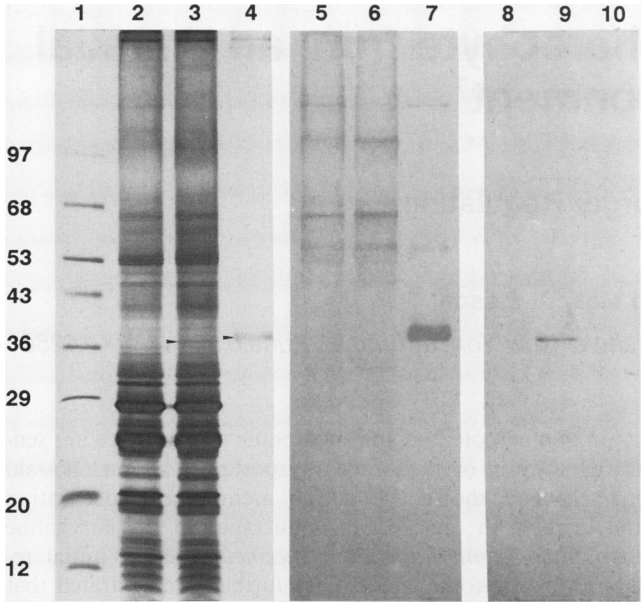

Figure 6. Silver-stain (lanes 1–4) and Western blots (lanes 5–10) of SDS-PAGE gel of membrane fractions from CO_2-enriched cells (lanes 2, 5, and 8) and air-adapted cells (lanes 3, 6, and 9) and concentrated external protein from air-adapted cells (lanes 4, 7, and 10) of *C. reinhardtii*. Lanes 5–7 were probed with antiperiplasmic carbonic anhydrase antiserum diluted 1:500 and lanes 8 to 10 were probed with anti-LIP-36 antiserum diluted 1:100. Lane 1 contains molecular size markers. Arrows indicate LIP-36 in lane 3 and periplasmic carbonic anhydrase in lane 4.

it is found exclusively in the membrane fraction, whereas the periplasmic carbonic anhydrase is a soluble protein which is excreted into the medium in cell wall-less mutants. In addition, the primary translation product of the periplasmic carbonic anhydrase was reported to be approximately 44 kD (5), while that of LIP-36 was found to be only 36 kD. More conclusively, it was shown that there was no immunological cross-reactivity between the two proteins when antibodies raised against LIP-36 and periplasmic carbonic anhydrase were used.

Its association with non-thylakoid membranes and the correlation of its appearance with C_i accumulation are consistent with the possible involvement of LIP-36 in C_i transport. These indications are by no means conclusive, however, as a 42 kD polypeptide with similar characteristics was identified in the cyanobacterium *Anacystis nidulans* and thought to be an essential component of C_i transport (11). It was subsequently shown, following isolation of the structural gene for the protein and construction of a mutant totally deficient in the protein, that this was not the case (13). Nevertheless, the known characteristics of LIP-36 make it a candidate for involvement in the CO_2-concentrating system until proven otherwise.

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