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Cloning and Overexpression of Two cDNAs Encoding the Low-CO_2-Inducible Chloroplast Envelope Protein LIP-36 from *Chlamydomonas reinhardtii* [1]

Zhi-Yuan Chen[2], Lara L. Lavigne, Catherine B. Mason, and James V. Moroney*

Department of Plant Biology, Louisiana State University, Baton Rouge, Louisiana 70803

***Chlamydomonas reinhardtii*, a unicellular green alga, grows photoautotrophically at very low concentrations of inorganic carbon due to the presence of an inducible CO_2-concentrating mechanism. During the induction of the CO_2-concentrating mechanism at low-CO_2 growth conditions, at least five polypeptides that are either absent or present in low amounts in cells grown on high-CO_2 concentrations are induced. One of these induced polypeptides with a molecular mass of 36 kD, LIP-36, has been localized to the chloroplast envelope. The protein was purified and the partial internal amino acid sequences were obtained through lys-C digestion. Two cDNAs encoding LIP-36 have been cloned using degenerate primers based on the amino acid sequences. The two genes encoding LIP-36 are highly homologous in the coding region but are completely different in the 5′-end and 3′-end untranslated regions. The deduced protein sequences show strong homology to the mitochondrial carrier protein superfamily, suggesting that LIP-36 is a chloroplast carrier protein. The regulation of the expression of these two genes at high- and low-CO_2 growth conditions is also different. Both genes were highly expressed under low-CO_2 growth conditions, with the steady-state level of LIP-36 G1 mRNA more abundant. However, neither gene was expressed at high-CO_2 growth conditions. The gene products of both clones expressed in *Escherichia coli* were recognized by an antibody raised against LIP-36, confirming that the two cDNAs indeed encode the *C. reinhardtii* chloroplast envelope carrier protein LIP-36.**

Many algal species, including the green alga *Chlamydomonas reinhardtii*, exhibit decreased photorespiration and an increased affinity for CO_2 when grown on limiting (≤355 parts per million) CO_2 levels than when grown at elevated (5%) CO_2 concentrations (Badger et al., 1980). In *C. reinhardtii*, these physiological changes are due to the induction of a CCM that accumulates C_i within the cell (Badger et al., 1980; Moroney and Mason, 1991). At low-CO_2 growth conditions, when the cell induces the CCM and increases its affinity for external C_i, at least five polypeptides that are either absent or present in low amounts in cells grown on high-CO_2 concentrations are synthesized (Coleman and Grossman, 1984; Manuel and Moroney, 1988; Spalding and Jeffrey, 1989). Several of these polypeptides are absent in various mutants that do not adapt to low CO_2, suggesting a role for these polypeptides in the CCM (Manuel and Moroney, 1988; Moroney et al., 1989).

One of the low-CO_2-inducible proteins with a molecular mass of 36 kD, LIP-36, was found to partition with the membrane fraction (Spalding and Jeffrey, 1989). In another study, Geraghty et al. (1990) raised an antibody to LIP-36 and demonstrated that this protein was immunologically distinct from the periplasmic carbonic anhydrase, which has a molecular mass of 37 kD. In addition, Mason et al. (1990), using ^{35}S labeling of *C. reinhardtii* proteins, showed that LIP-36 was localized to intact chloroplasts. Recent investigations (Ramazanov et al., 1993) specifically localized this polypeptide to chloroplast envelope membranes isolated from low-CO_2-grown cells and demonstrated that it is not present in chloroplast envelopes isolated from high (5% CO_2 in air [v/v])-CO_2-grown cells. This 36-kD protein does not show carbonic anhydrase activity and was not present on plasma membranes isolated from low-CO_2-grown cells. However, the identity and function of this chloroplast envelope protein were unknown. In this paper we report the molecular cloning of two cDNAs encoding LIP-36, their partial characterization, and the overexpression of these proteins in *Escherichia coli*. LIP-36 may, in part, account for the different C_i uptake characteristics observed in chloroplasts isolated from high- and low-CO_2-grown cells of *C. reinhardtii* (Moroney and Mason, 1991).

MATERIALS AND METHODS

Algal Strains and Culture Conditions

The cell wall-deficient mutant of *Chlamydomonas reinhardtii*, CC-400 (cw-15 mt^+), was obtained from the Duke University *Chlamydomonas* Culture Collection. Wild-type cells (137 mt^+) were obtained from Dr. R.K. Togasaki and maintained in our laboratory. In liquid culture, the cells were grown in minimal medium, aerated with air or air supplemented with 5% CO_2 (v/v), and illuminated with 300 $\mu E\ m^{-2}\ s^{-1}$ of white light. For chloroplast envelope preparations, cells were grown synchronously with 12-h

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[2] Present address: Southern Regional Research Center, U.S. Department of Agriculture-Agricultural Research Service, 1100 Robert E. Lee Boulevard, New Orleans, LA 70124.

* Corresponding author; e-mail btmoro@unix1.sncc.lsu.edu; fax 1–504–388–8459.

Abbreviations: CCM, CO_2-concentrating mechanism; C_i, inorganic carbon; IPTG, isopropylthio-β-galactoside.

light/12-h dark cycles and harvested 5 h into the light cycle.

Isolation of LIP-36 Protein from the Chloroplast Envelope

All steps were carried out at 0 to 4°C. Intact chloroplasts were isolated as described by Mason et al. (1991). Chloroplast envelopes were isolated by modification of the method of Clemetson and Boschetti (1988) as described by Ramazanov et al. (1993). The chloroplast membranes were then resuspended in 100 μL of resuspension buffer, pH 7.8 (10 mM Tris, 1 mM Na_2-EDTA, and 10 μM leupeptin).

The chloroplast envelopes isolated from air-grown CC-400 cw-15 cells were subjected to 12% SDS-PAGE with 0.1% thioglycolate in the upper buffer. The envelope proteins were then transferred to Immobilon (Millipore), and the Immobilon was stained with acid-free Coomassie blue (50% methanol, 2.5% Coomassie blue R-250) and destained with 50% methanol. The band corresponding to LIP-36 protein was cut out for lys-C digestion and amino acid sequencing at the Baylor Medical School protein sequencing facility (Houston, TX). Approximately 100 pmol of LIP-36 protein was recovered from the membrane.

Cloning of the cDNAs Encoding LIP-36

Two degenerate primers, B-1 (5′-CCRCCRTARATRTGYTC) and F-3 (5′-TIGARGGIGAYGAYCAYTA), were made based on the amino acid sequences of peptides 1 ([W/R]YEENVEHIYGGVIGPAT?TAAQ) and 3 (LEGDDHYSNFSHANVLLSGAAA) and using the codon bias of *C. reinhardtii*. One microgram of poly(A)$^+$-enriched mRNA purified from wild-type *C. reinhardtii* cells that had adapted to low-CO_2 growth conditions for 2 h was reverse transcribed in 20 μL of volume with Rs+dT primer (5′-GAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTT-3′) at 45°C for 1 h using SuperScript II RNase H$^-$ reverse transcriptase (GIBCO-BRL) as previously described (Chen et al., 1996). The following PCR components were assembled on ice in a 0.5-mL tube: 2.5 μL of 20× reaction buffer (1.0 M Tris-HCl, pH 9.0, 0.4 M ammonium sulfate), 4 μL of 25 mM $MgCl_2$, 3 μL of 2.5 mM (each) dNTP, 3 μL of 10 μM primer B-1, 3 μL of 10 μM primer F-3, 0.5 μL of formamide, 1 μL of 1:20-diluted first-strand cDNA, 33 μL of water, and 0.3 unit of *Tfl* DNA polymerase (Epicenter, Madison, WI). The PCR profiles used to amplify the cDNA encoding LIP-36 were: 95°C for 40 s, 48°C for 1 min, and 72°C for 2 min for 35 cycles, and 1 cycle at 72°C for 15 min. The amplified PCR product was subsequently cloned into pCRII vector and named LIP-36 M.

The 5′ end and 3′ end of the cDNAs encoding LIP-36 were cloned using a modified 5′ rapid amplification of cDNA ends procedure. For the cloning of the 5′ end of the LIP-36 cDNAs, mRNA was reverse transcribed with B-1 primer, and the first-strand cDNA was tailed with poly(A)$^+$. The primer sets used for first- and second-round PCR were Rs+dT and B-1 (CCRCCRTARATRTGYCAYTA) and Rs+dT and B-4 (5′-GGATGGCACGGTACATGG), respectively. For the cloning of the 3′ end of the LIP-36 cDNAs, mRNA was reverse transcribed with the Rs+dT primer. The primer sets used for first- and second-round PCR were Rs+dT and F-3 and Rs+dT and F-5 (5′-CCATGTACCGTGCCATCC), respectively. The PCR-amplified DNA fragments were then cloned into pCRII vector (named LIP-36 L for the 5′ end clone and LIP-36 R for the 3′ end clone) for DNA sequence analysis.

Northern Analysis

RNA samples isolated at different times from wild-type (137 mt$^+$) cells adapting to low CO_2 were prepared, electrophoresed, and blotted as previously described (Chen et al., 1996). The blots were probed with inserts from LIP-36 M clones as probes to study whether the corresponding mRNA is up-regulated at low-CO_2 growth conditions. For the study of differential expressions of LIP-36 gene 1 (G1) and gene 2 (G2) at mRNA level, the gene-specific fragments corresponding to the 3′ noncoding region amplified from PCR were used as probes. For LIP-36 G1, the probe corresponds to the region between bp 1338 and 1970 and was amplified using primers F1-A (5′-TGACAACATGGCTCAAAAGGTG) and B1-A (5′-GATGGAGAAACAGACCAGCA). For LIP-36 G2, the probe corresponds to the region between bp 1344 and 2013 and was amplified using primers F2-A (5′-TACACGCTTTGTAGTGTTGTTTC) and B2-A (5′-GTGTCTCCTCCTTGCCTG). Northern analysis was performed as described by Sambrook et al. (1989).

DNA Sequencing and Sequence Homology Analysis

Plasmid DNAs were prepared using Qiagen (Chatsworth, CA) spin columns from 2 mL of overnight culture according to the manufacturer's instructions. For DNA sequencing the Perkin-Elmer Amplicycle sequencing kit was used. Sequence comparisons were made using the BLAST (Altshul et al., 1990) or BLITZ (Biocomputing Research Unit, Edinburgh, UK) programs via e-mail servers.

Construction of the Expression Plasmids

Primers G1–5′ END (5′-GTCCACAAGCGAATTCTGGATATC) and CLN14 (5′-AAGCAGAAAGCTTACGACCACATCTA) were used to amplify the LIP-36 gene 1 coding region, which corresponds to the segment between 216 and 1446 bp, with two nucleotide changes (underlined). One change was made at position 227 bp (from C to A) to incorporate an *Eco*RI restriction site at the 5′ end of the fragment, and the other was made at position 1430 bp (from A to T) to incorporate a *Hin*dIII site at the 3′ end of the fragment. Primers G2–5′ END (5′-GTCCACAAGCGAATTCTCGATATC) and CLN5 (5′- GCAAGCTCAAGCTTTCCCTCACCCCA) were used to amplify the LIP-36 gene 2 coding region, which corresponds to the segment between 209 and 1585 bp, with two nucleotide changes (underlined). One change was made at position 220 bp (from C to A) to incorporate an *Eco*RI restriction site at the 5′ end of the fragment, and the other was made at position 1572 bp (from A to T) to incorporate a *Hin*dIII site at the 3′ end of the fragment.

The PCR-amplified DNA fragments were purified using Qiagen columns and digested with *Eco*RI and *Hin*dIII overnight. The digested PCR products corresponding to the regions between 227 and 1434 bp for LIP-36 G1 and between 220 and 1576 bp for LIP-36 G2 were then separately ligated into the vector pET28-c (Kan, Novagen, Madison, WI). After transformation of the ligation mixtures into *E. coli* strain DH5α, positive transformants were identified by PCR amplification of the inserts according to the manufacturer's instructions. One plasmid, containing the insert corresponding to amino acid sequences from residues 24 to 358 from LIP-36 G1, was named pet.-G1; and the other plasmid, containing the insert from LIP-36 G2 corresponding to amino acid sequences from residues 24 to 355, was named pet.-G2.

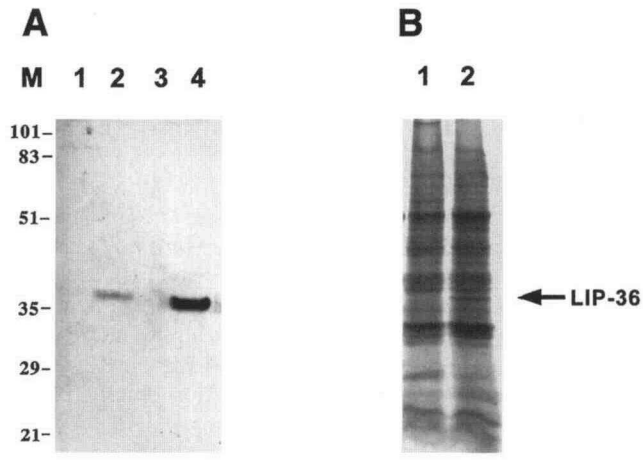
Bacterial Expression and Isolation of LIP-36

Plasmid DNAs of pet.-G1 and pet.-G2 were used to transform the expression host cells *Escherichia coli* BL21(DE3) (Novagen). A single colony was picked from each transformation and inoculated into 2 mL of Luria broth medium (with 30 μg/mL Kanamycin and incubated with shaking at 37°C overnight. The cells were then transferred to a flask with 50 mL of Luria broth plus Kanamycin medium. The incubation was continued for another 2 h until the A_{600} reached 0.4. Aliquots were removed for the uninduced controls, and IPTG was added to the remaining medium to a final concentration of 1 mM to induce the target gene expression. The cells were incubated for another 3 h. The flask was placed on ice for 5 min and then the cells were harvested by centrifugation at 5,000*g* for 5 min at 4°C. The cells were resuspended in a 25% culture volume of cold 50 mM Tris-HCl, pH 8.0, 2 mM EDTA and centrifuged as above. The pellet was resuspended in 0.6 mL of 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 0.2 mL of the resuspended cells was sonicated for 2 min and centrifuged at 15,000 rpm for 10 min. The supernatant was kept as total soluble protein. The pellet, which contained mainly inclusion bodies, was washed and centrifuged once as above and saved as insoluble protein, which was solubilized in 0.5% Sarkosyl, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA.

RESULTS

Isolation of LIP-36 from the Chloroplast Envelope of Low-CO_2-Grown Cells

LIP-36 was previously localized to the chloroplast envelope (Ramazanov et al., 1993). To isolate enough of this protein for sequencing, 8 L of a low-CO_2-adapted *C. reinhardtii* culture (strain CC-400) was harvested. The induction of LIP-36 protein was examined by western analysis before isolation (Fig. 1A). The immunoblot showed that this protein is specifically induced at low-CO_2 growth conditions. It is also very clear that LIP-36 is present in the chloroplast envelopes of low-CO_2-grown cells, whereas it is missing in the chloroplast envelopes of high-CO_2-grown cells (Fig. 1B). The chloroplast envelopes isolated from high- and low-CO_2-grown cells were resolved on SDS-PAGE and transferred to Immobilon. The membrane was



C

Peptide 1: (R/W)YEENVEHIYGGVIGPATT(?)AAQ
Peptide 2: (S?)ILDILPGISGGVAIVMIG
Peptide 3: (?)LEGDD(H/D)Y(S?)NFSSANVLL(?)GAAAG
Peptide 4: (S/V)(E/R)(G/L)(Q/P)(V/L)(L/S)(G/F)(Q/Y)(G/K)TALAAK

Figure 1. The induction of LIP-36 protein at high- and low-CO_2 growth conditions. A, Western analysis of the induction of LIP-36 protein at low-CO_2 growth conditions. Fifty micrograms of total cell proteins (lanes 1 and 2) or 10 μg of isolated chloroplast envelope proteins (lanes 3 and 4) from high-CO_2- (lanes 1 and 3) and low-CO_2-grown cells (lanes 2 and 4) was blotted to nitrocellulose and probed with LIP-36 antibodies. In lane M, the relative molecular masses of standard proteins are given in kD. B, SDS-PAGE of the chloroplast envelopes isolated from high- and low-CO_2-grown cells stained with Coomassie blue. Fifty micrograms of chloroplast envelopes from high (lane 1) and low CO_2 (lane 2) was loaded onto the gel. The arrow indicates LIP-36, which is present only in the chloroplast envelope of low-CO_2-grown cells. C, Partial protein sequences obtained from four major peptides of lys-C-digested LIP-36 protein. Note that peptide 4 was a mixture of two different peptides.

then subjected to acid-free Coomassie blue staining to visualize LIP-36. The protein band corresponding to LIP-36 was cut out for protein sequence analysis. After lys-C digestion and separation of the peptides by HPLC, four major peptides were sequenced (Fig. 1C).

Cloning of the Genes Encoding LIP-36

Based on the amino acid sequences of peptides 1 and 3 (Fig. 1C), two sets (forward and reverse) of degenerate primers were made and used to amplify the corresponding gene product by PCR using reverse-transcribed mRNA as a template. The PCR mixture was resolved on a 0.8% agarose gel. Only the F-3 (forward) and B-1 (reverse) primers specifically amplified a 0.7-kb DNA band.

The PCR-amplified 0.7-kb DNA was then recovered and subsequently cloned into pCRII cloning vector (Invitrogen, San Diego, CA) according to the manufacturer's instructions. All of the positive clones had the same size inserts (0.7 kb) and were named LIP-36 M (Fig. 2). Seven indepen-

dent clones were used for DNA sequence analysis. DNA sequences of the cloned LIP-36 M matched the known peptide sequences, which confirmed that the PCR product is part of the gene encoding LIP-36. An interesting observation was that the DNA sequences of the seven clones examined were found to belong to two groups. Five of the clones have the same DNA sequence, and although DNA sequences of the other two clones were very homologous to the sequences of those five clones, they were not identical to them. This suggests that LIP-36 is encoded by two genes. Clones sharing the same DNA sequences as those five were designated as LIP-36 G1, and clones sharing the same DNA sequences as the other two were named LIP-36 G2.

Based on the DNA sequence information obtained from LIP-36 M, the 5′ end and 3′ end of the cDNAs encoding LIP-36 were cloned as described in "Materials and Methods." Using 5′ rapid amplification of cDNA ends, the 1.2-kb 5′ end and the 0.8-kb 3′ end of the cDNAs encoding LIP-36 were amplified and cloned into the pCRII vector. The clones harboring the 1.2-kb 5′ end PCR products were named LIP-36 L, and those having the 0.8-kb insert were named LIP-36 R. The restriction map of the two cDNA clones encoding LIP-36 and the relative positions of the clones LIP-36 M, LIP-36 L, and LIP-36 R are shown in Figure 2.

DNA Sequencing of the Two Genes Encoding LIP-36

Five to 10 independent clones each from of LIP-36 M, LIP-36 L, and LIP-36 R were sequenced. Two different 5′ ends and two different 3′ ends were found, which confirmed that LIP-36 is encoded by two genes. An interesting discovery was that the 3′ end DNA sequence of LIP-36 G1 is identical to one of our low-CO_2-inducible clones, 4I7, previously selected by differential screening (Burow et al., 1996). The DNA sequences of LIP-36 G1 and LIP-36 G2 were obtained by merging the DNA sequences from corresponding LIP-36 M, LIP-36 L, and LIP-36 R clones. The complete DNA sequence of LIP-36 G1 is 2025 bp and that of LIP-36 G2 is 2068 bp, which agree with the mRNA size estimated from northern analysis. LIP-36 G1 has an open reading frame of between 156 and 1232 bp with the potential for encoding a polypeptide of 358 amino acids. The open reading frame for LIP-36 G2 is 3 amino acids shorter. It has the potential for encoding a polypeptide of 355 amino acids, with the start codon at 149 bp and the stop codon at 1216 bp. A comparison of the DNA sequences between the two genes shows that the noncoding regions are much less homologous (40% identity) to each other, whereas the coding regions share 96.5% identity. The homology of the deduced amino acid sequence between the two genes was 95.7% (Fig. 3), with only 14 amino acid substitutions in the entire coding region between LIP-36 G1 and LIP-36 G2.

The calculated molecular weights for the gene products of LIP-36 G1 and LIP-36 G2 are 38,345 and 38,049, respectively, whereas the molecular weight of LIP-36 as determined by SDS-PAGE is 36,000. This difference is possibly due to the presence of a potential transit sequence, which is required to direct the gene product to the chloroplast envelope. It is clear that the mature protein contains at least residue 23 (Lys), as inferred from peptide sequence results (Fig. 1C). By comparing the N-terminal sequences of LIP-36 G1 and LIP-36 G2 with known *C. reinhardtii* chloroplast

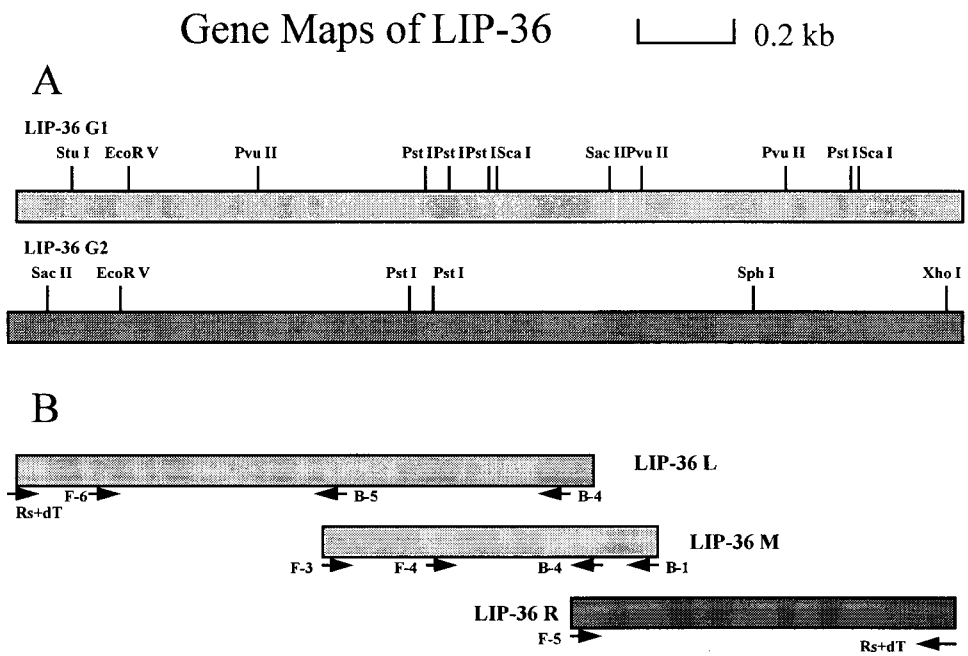


Figure 2. Restriction map of the two genes encoding LIP-36 (A) and the relative positions of clones LIP-36 M, LIP-36 L, and LIP-36 R (B). Primers Rs+dT, B-1, and B-4 were used to clone the 5′ end of the cDNAs, whereas primers Rs+dT, F-3, and F-5 were used to clone the 3′ ends of the cDNAs (the primer sequences are given in "Materials and Methods"). Primers B-5, F-4, and F-6 were used to sequence the clones.

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LIP-36 G1      MSSDAMTINESLMEV EHTPAVHKRILDILP GISGGVARVMIGQPF DTIKVRLQVLGQGTA  60
LIP-36 G2      MASDAMTINEALMEV EHTPAVHKRILDILP GISGGVARVMIGQPF DTIKVRLQVLGQGTA  60
                *       *

               LAAKLPPSEVYKDSM DCIRKMVKSEGPLSF YKGTVAPLVGNMVLL GIHFPVFSAVRKQLE 120
               LAAKLPPSEVYKDSM DCVRKMIKSEGPLSF YKGTVAPLVGNMVLL GIHFPVFSSVRKMLE 120
                                 *  *                          *   *

               GDDHYSNFSHANVLL SGAAAGAAGSLISAP VELVRTKMQMQRRAA LAGTVAAGAAASAGA 180
               GDDHYSNFSHANVLL SGAAAGAAGSLISAP VELVRTKMQMQRRAA LAGTVAAGAAASAGA 180


               EEFYKGSLDCFKQVM SKHGIKGLYRGFTST ILRDMQGYAWFFLGY EATVNHFLQNAGPGV 240
               EEFYKGSLDCFKQVM SKHGIKGLYRGFSST ILRDMQGYAWFFLGY EATVNHFLQNAGPGV 240
                                          *


               HTKADLNYLQVMAAG VVAGFGLWGSMFPID TIKSKLQADSFAKPQ YSSTMDCLKKVLASE 300
               HTKADLNYLQVMAAG VVAGFGLWGSMFPID TIKSKMQADSFVKPQ YSTTIDCVKQVIASE 300
                                             *     *      * *  * * *


               GQAGLWRGFSAAMYR AIPVNAGIFLAVEGT RQGIKWYEENVEHIY GGVIGPATPTAAQ   358
               GQAGLWRGFSAAMYR AIPVNAGIFLAVEGT RQGIKWYEENVEHIY GGVIGPAT---AQ   355
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Figure 3. The deduced amino acid sequences of the two genes encoding LIP-36. The substitutions in amino acid sequences between the two genes are indicated by asterisks. The regions that match the known peptide sequences are underlined. The three mitochondrial carrier protein superfamily consensus repeats are indicated by boldface letters.

proteins (Franzén et al., 1990), it appears likely that the N-terminal sequence before residue 21 (Val) is clipped off when the protein is targeted to the chloroplast envelope.

Sequence Homology Analysis of LIP-36

The homology search (at http://dot.imgen.bcm.tmc.edu) using the deduced amino acid sequences found that LIP-36 protein has high homology (30- 35%) to proteins of the mitochondrial carrier protein superfamily, such as yeast mitochondrial carrier protein 1 (YMC-1) and 2 (YMC-2) (Fig. 4). The common characteristic of established members of the mitochondrial carrier protein superfamily is that their polypeptide chains consist of three tandemly repeated related sequences of approximately 100 amino acids (Saraste and Walker, 1982). Each of these three repeated elements is probably folded into two transmembrane α-helices linked by an extensive polar region, forming a structure with six transmembrane α-helices. The consensus pattern of proteins belonging to this family

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1 YMC-1        MSEEFPSPQLIDDLE ---------EHPQHD NARVVKDLLAGTAGG IAQVLVGQPFDTTKV     51
2 YMC-2        MSEEFPTPQLLDELE DQQKVTTPNEKRELS SNRVLKDIFAGTIGG IAQVLVGQPFDTTKV     60
3 LIP-36G1     ---------MSSDAM TINESLMEVEHTPAV HKRIL-DILPGISGG VARVMIGQPFDTIKV     50
                                 *          *   *    * **  * * ******* **


               RLQTSSTPTT----- --------AMEVVRK LLANEGPRGFYKGTL TPLIGVGACVSLQFG     98
               RLQTATTRTT----- --------TLEVLRN LVKNEGVFAFYKGAL TPLLGVGICVSVQFG    107
               RLQVLGQGTALAAKL PPSEVYKDSMDCIRK MVKSEGPLSFYKGTV APLVGNMVLLGIHFP    110
               ***                                **   ****    ** *


               VNQAMKRFFHHRNA- ---DMSS-------- -TLSLPQYYACGVTG GIVNSFLASPIEHVR    145
               VNEAMKRFFQNYNAS KNPNMSSQDVDLSRS NTLPLSQYYVCGLTG GVVNSFLASPIEQIR    167
               VFSAVRK--QLEGDD HYSNFSHANVLLS-- -----------GAAA GAAGSLISAPVELVR    155
               *               *                          *   *   *   * * *


               IRLQTQ--------- -----TGSGTNAEFK GPLECIKKLRHN--- KALLRGLTPTILREG    188
               IRLQTQ--------- -----TSNGGDREFK GPWDCIKKLKAQ--- GGLMRGLFPTMIRAG    210
               TKMQMQRRAALAGTV AAGAAASAGAEEFYK GSLDCFKQVMSKHGI KGLYRGFTSTILRDM    215
                  * *                  *    * *   * *          * **   *  *


               HGCGTYFLVYEALIA NQM-NKRRGL-ERKD IPAWKLCIFGALSGT ALWLMVYPLDVIKSV    246
               HGLGTYFLVYEALVA REI-GT--GL-TRNE IPPWKLCLFGAFSGT MLWLTVYPLDVVKSI    266
               QGYAWFFLGYEATVN HFLQNAGPGVHTKAD LNYLQVMAAGVVAGF GLWGSMFPIDTIKSK    275
                *    ** ***           *                 *  *   **    * *  **


               MQTDNLQKPKFGNSI SSVAKTLYANGGIGA FFKGFGPTMLRAAPA NGATFATFELAMRLL    306
               IQNDDLRKPKYKNSI SYVAKTIYAKEGIRA FFKGFGPTMVRSAPV NGATFLTFELVMRFL    326
               LQADSFAKPQYSSTM DCLKKVL-ASEGQAG LWRGFSAAMYRAIPV NAGIFLAVEGTRQGI    334
                * *   **          *   *  *       **   * * *   *   *   *


               G-------------- ---------    307
               G--EE---------- ---------    329
               KWYEENVEHIYGGVI GPATPTAAQ    358
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Figure 4. Sequence homology analysis of the deduced amino acid sequence of LIP-36 G1 with the yeast mitochondrial carrier proteins YMC-1 and YMC-2. The conserved amino acid residues are indicated with asterisks, and gaps are indicated with dashes.

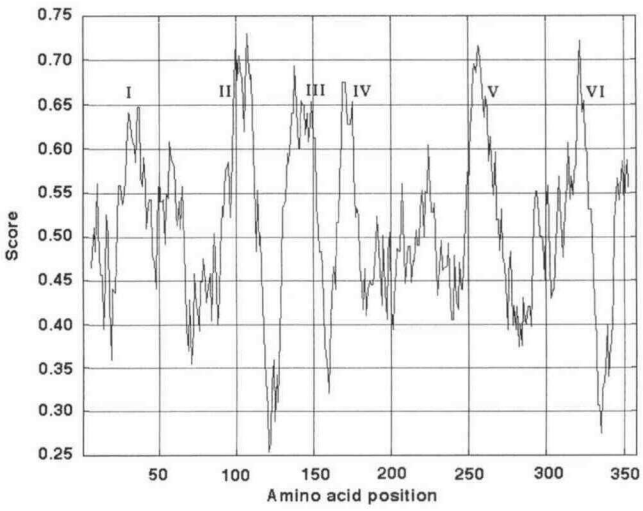

Figure 5. The hydropathy profile of the deduced amino acid sequence of LIP-36 G1. The window size is 11 amino acids, and the possible transmembrane regions are indicated with Roman numerals I to VI. The algorithm used was *TMBASE* (Hofmann and Stoffel, 1993).

(PS00215, PDOC00189 in SWISS-PROT) generated by selecting one of the most conserved regions in the repeated domain located just after the first transmembrane region is P-X-[DE]-X-[LIVAT]-[RK]-X-[LRH]-[LIVMFY]. This consensus sequence is present in LIP-36 encoded by either cDNA, and is repeated three times from amino acid residue 44 to 52, 150 to 158, and 268 to 276 (Fig. 3).

The hydrophobicity distribution analysis of the deduced amino acid sequences of LIP-36 using the ExPASy online sequence-analysis program also indicated the presence of six possible membrane-spanning α-helices (Fig. 5). This analysis is in general agreement with the hydropathy profiles of members of a mitochondrial carrier protein superfamily of known function that have been sequenced (Kaplan et al., 1995). It is noteworthy that (a) the N-terminal region (the first 20 amino acid residues) that constitutes the putative chloroplast transit sequence is more hydrophilic and may contain a small α-helix between residues 8 and 17, and (b) the hydropathy profile suggests that the C terminus of *C. reinhardtii* LIP-36 is probably not embedded in the chloroplast membrane. Based on these results, LIP-36 is likely to be a chloroplast envelope-located carrier protein.

Induction of Gene Expression at Low-CO_2 Growth Conditions

The presence of two genes encoding the same protein, LIP-36, suggests that they may be regulated differently by CO_2. To determine whether this is the case, total RNA was isolated from CO_2-grown cells and cells that had been adapted to air for 1, 2, 4, 6, and 10 h. The expression of LIP-36 G1 and LIP-36 G2 in high- and low-CO_2-grown cells was compared by using gene-specific probes to probe two identical northern blots. The probes were prepared by labeling the PCR-amplified 3′ end noncoding region using primer sets F1-A and B1-A for the LIP-36 G1 probe and F2-A and B2-A for the LIP-36 G2 probe. The mRNAs of LIP-36 G1 and LIP-36 G2 were undetectable in cells grown at high-CO_2 growth conditions. In contrast, the steady-state levels of both mRNAs dramatically increased and remained high after cells were switched from high-CO_2 to low-CO_2 growth conditions. However, the pattern of induction is slightly different between the two genes (Fig. 6). The mRNA of LIP-36 G2 appeared to respond to the change in CO_2 concentration faster than that of LIP-36 G1. The mRNA of LIP-36 G1 was still undetectable 1 h after the cells were switched to low-CO_2 conditions, whereas that of LIP-36 G2 was present at low concentrations. In addition, the steady-state mRNA level of LIP-36 G1 in low-CO_2-grown cells (time $>$ 2 h) was at least two times more abundant than that of LIP-36 G2, which qualitatively agrees with the recovery of more LIP-36 G1 clones from the reverse transcription and PCR cloning experiments described earlier.

Overexpression of LIP-36 Protein in *E. coli*

To verify that the two cDNA clones are really encoding LIP-36, both genes were cloned into an *E. coli* expression vector, and the overexpressed proteins were analyzed by western blots. The DNA fragments that equal the region between 227 and 1434 bp for LIP-36 G1 and that between 220 and 1576 bp for LIP-36 G2 were separately fused into corresponding sites in the expression vector pET-28c (Kan)

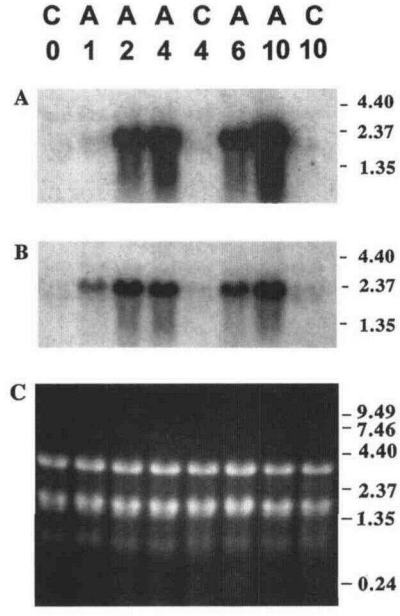

Figure 6. Northern analysis showing the differential expression of the two genes at high- and low-CO_2 growth conditions. The high-CO_2-grown cells were switched to low-CO_2 conditions at 0 h. Total RNA was isolated at 0, 4, and 10 h from cells left growing under high-CO_2 conditions (lanes C). Total RNA was also isolated from low-CO_2-adapting cells at 1, 2, 4, 6, and 10 h (lanes A). A, The expression of LIP-36 G1. B, The expression of LIP-36 G2. C, Ethidium bromide-stained gel showing the RNA loads. Ten micrograms of total RNA was loaded per lane.

as described in "Materials and Methods." The expression of LIP-36 was induced by adding IPTG to 1.0 mM when the *E. coli* expression host BL21(DE3) cells containing either pet.-G1 or pet.-G2 reached an A_{600} of 0.4. As expected, the expressed LIP-36 protein was found to be highly insoluble, and it was enriched in the inclusion bodies (Fig. 7A). The overexpressed protein accounts for 30% of the total cell insoluble protein for both constructs (Fig. 7A). In addition, both of the overexpressed proteins were recognized by the antibodies raised against LIP-36 (Fig. 7B), thus confirming that the two cDNA clones are indeed encoding the low-CO_2-inducible chloroplast envelope protein LIP-36.

DISCUSSION

Although the existence of a CCM in *Chlamydomonas* spp. has been demonstrated, no components other than the periplasmic carbonic anhydrase have been identified. Proteins such as LIP-36 (Spalding and Jeffrey, 1989; Geraghty et al., 1990; Mason et al., 1990), the synthesis of which is correlated with the induction of the CCM, could be components of the 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 the chloroplast envelope of air-growing cells but not high-CO_2-grown cells (Ramazanov et al., 1993), which suggests that it could be a transporter protein responding to low CO_2 levels.

The LIP-36 protein was isolated by resolving the chloroplast envelope proteins isolated from low-CO_2- and high-CO_2-grown cells, transferring the proteins to Immobilon,

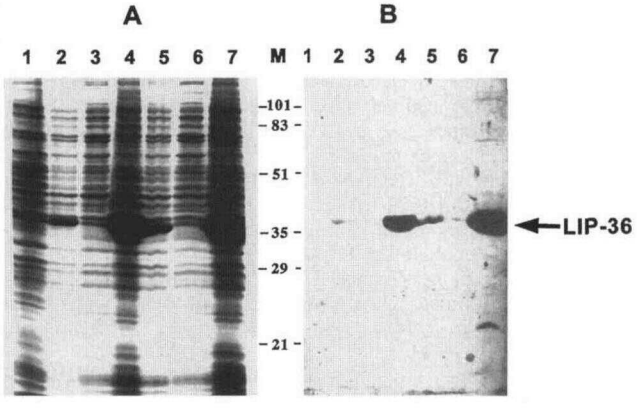

Figure 7. Overexpression of LIP-36 in *E. coli*. The LIP-36 gene 1 coding region corresponding to amino acid sequence 24 to end (residue 358) and the gene 2 coding region corresponding to amino acid sequence 24 to end (residue 355) were cloned into pET-28c. LIP-36 protein was overexpressed from both genes (lanes 1–4, gene 1; lanes 5–7, gene 2) upon induction with IPTG. Lanes 1, Total proteins from uninduced *E. coli* cells; lanes 2 and 5, total protein from induced *E. coli* cells; lanes 3 and 6, the soluble protein fraction from induced *E. coli* cells; lanes 4 and 7, the insoluble protein fraction from induced *E. coli* cells; lane M, prestained molecular mass marker (Bio-Rad) indicating relative molecular masses in kD. A, SDS-PAGE stained with Coomassie blue. B, Western analysis of the same gel probed with LIP-36 antibody.

and cutting out the low-CO_2-inducible protein band with a molecular mass of approximately 36 kD. Two cDNAs encoding LIP-36 were cloned using degenerate primers, and the remainder of the cDNA sequence was obtained using 5′ rapid amplification of cDNA ends. After the initial PCR amplification of the LIP-36 M, a northern analysis was performed that demonstrated that the message was present only in low-CO_2-grown cells, not high-CO_2-grown cells, and that the size of the message recognized by LIP-36 M was about 2.0 kb (data not shown). Both cloned cDNAs had long 3′ untranslated regions, which is consistent with what is known for other low-CO_2-inducible cDNAs (Fukuzawa et al., 1990; Burow et al., 1996). The deduced amino acid sequences of the two cDNA clones were found to have all of the five peptide sequences (Fig. 3), with only a couple of amino acid sequence disagreements, which were later confirmed to have arisen from peptide sequencing. To confirm that the two genes cloned are indeed encoding LIP-36, both of the cloned genes were overexpressed in *E. coli*, and both overexpressed proteins were recognized by the LIP-36 antibodies (Fig. 7B).

Regulation of Gene Expression

The presence of two genes encoding LIP-36 suggests that they may be regulated differently by CO_2, with one serving as a constitutive gene and the other as a low-CO_2-inducible gene, just like other studied low-CO_2-inducible genes, such as *Cah1* and *Cah2* (Fujiwara et al., 1990; Fukuzawa et al., 1990). Actually, this is not the case, even though the expression of the two genes is slightly different. In low-CO_2-grown cells, the mRNA level of LIP-36 G1 is more abundant than that of LIP-36 G2, but LIP-36 G2 appears to respond to the decrease in CO_2 levels faster than LIP-36 G1 (Fig. 6). However, neither of the messages was detected in high-CO_2-grown cells (Fig. 6). After cells are switched to low CO_2, the LIP-36 messages of both increase in abundance. The mRNA for LIP-36 G2 was detectable in cells adapted to low CO_2 after only 1 h, whereas the mRNA for LIP-36 G1 was detected only in cells adapted to low CO_2 for 2 h or longer (Fig. 6). These results are consistent with earlier studies (Geraghty et al., 1990; Ramazanov et al., 1993) in which LIP-36 antibodies were able to detect low levels present at 2 h and substantial levels were reached by 8 h. The time course of the induction of these messages is also similar to the time-course of induction of both increased photosynthetic rates and C_i accumulation, which is consistent with the possibility that LIP-36 is involved with C_i transport.

Homology Studies

The sequence homology search using the deduced amino acid sequences of LIP-36 found that both have significant homology to proteins that belong to the mitochondrial carrier protein superfamily. The highest homology was found between LIP-36 and YMC-1 (35.5%) (Fig. 4); unfortunately, the function of this carrier protein is still not clear. The common characteristic of established members of the mitochondrial carrier protein superfamily, the three tandemly

repeated sequences, is also present in the deduced amino acid sequence of LIP-36 (Figs. 4 and 5). Other proteins belonging to this family are the ATP/ADP carrier proteins from yeast, Arabidopsis, maize, and *C. reinhardtii*; the 2-oxoglutarate/malate transporter from a variety of organisms; uncoupling protein from brown fat cells; and the Brittle 1 protein from maize amyloplasts. One important point is that not all proteins in this family are mitochondrial proteins. For example, Brittle 1 has a chloroplast transit sequence and is located on the amyloplast membrane (Sullivan et al., 1991). Recently, a chloroplast envelope 2-oxoglutarate/malate transporter has also been described (Weber et al., 1995). Another example is the peroxisomal membrane protein PMP47 (Jank et al., 1993), which is an integral membrane protein of the peroxisome and may play a role as a transporter. A search of GenBank indicates that, so far, LIP-36 is one of the few chloroplast envelope transporters of either membrane that has genes that have been cloned. Other clones include the triose phosphate/phosphate translocator (Flügge et al., 1989), a Ca^{2+}-ATPase of the inner envelope membrane (Huang et al., 1993), a voltage-dependent anion channel (porin) of the outer envelope membrane (Fischer et al., 1994), components of the protein import apparatus, and the 2-oxoglutarate/malate transporter (Weber et al., 1995).

Overexpression and Possible Functions of LIP-36

The proteins in this family usually function as a homodimer and do not require prosthetic groups. If the expression of the carrier of unknown function can be achieved, as in the case of LIP-36, it would provide a number of potential routes to identify their transport specificities in the future, either by the reconstitution of the carrier into liposomes and the determination of their transport properties or by using the expressed proteins to facilitate the biochemical characterization of these carrier proteins. This methodology has been successfully used for several transporter proteins (Fiermonte et al., 1993; Kaplan et al., 1995; Weber et al., 1995).

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