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Zhi Yuan Chen  
*Louisiana State University*

Lara L. Lavigne  
*Louisiana State University*

Catherine B. Mason  
*Louisiana State University*

James V. Moroney  
*Louisiana State University*

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Cloning and Overexpression of Two cDNAs Encoding the Low-CO₂-Inducible Chloroplast Envelope Protein LIP-36 from Chlamydomonas reinhardtii

Zhi-Yuan Chen², 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 CO₂-concentrating mechanism. During the induction of the CO₂-concentrating mechanism at low-CO₂ growth conditions, at least five polypeptides that are either absent or present in low amounts in cells grown on high-CO₂ 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’- 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-CO₂ growth conditions is also different. Both genes were highly expressed under low-CO₂ growth conditions, with the steady-state level of LIP-36 G1 mRNA more abundant. However, neither gene was expressed at high-CO₂ 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 protein LIP-36.

Many algal species, including the green alga Chlamydomonas reinhardtii, exhibit decreased photorespiration and an increased affinity for CO₂ when grown on limiting (≤355 parts per million) CO₂ levels than when grown at elevated (5%) CO₂ concentrations (Badger et al., 1980). In C. reinhardtii, these physiological changes are due to the induction of a CCM that accumulates C₄ within the cell (Badger et al., 1980; Moroney and Mason, 1991). At low-CO₂ growth conditions, when the cell induces the CCM and increases its affinity for external C₄, at least five polypeptides that are either absent or present in low amounts in cells grown on high-CO₂ 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 CO₂, suggesting a role for these polypeptides in the CCM (Manuel and Moroney, 1988; Moroney et al., 1989).

One of the low-CO₂-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 35S 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-CO₂-grown cells and demonstrated that it is not present in chloroplast envelopes isolated from high (5% CO₂ in air [v/v])-CO₂-grown cells. This 36-kD protein does not show carbonic anhydrase activity and was not present on plasma membranes isolated from low-CO₂-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 C₄ uptake characteristics observed in chloroplasts isolated from high- and low-CO₂-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% CO₂ (v/v), and illuminated with 300 μE m⁻² s⁻¹ of white light. For chloroplast envelope preparations, cells were grown synchronously with 12-h
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 Na₂-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% thiglycolate 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 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'-CCRCRTARTATRTGYTC) and F-3 (5'-TIGARGGIGAYGAYG), were made based on the amino acid sequences of peptides 1 (IW/RIY)EENVEHYGGVIGPAT?TAAQ) and 3 (LEGG-DHYSNFSHAVNLSSGAA) 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-CO₂ growth conditions for 2 h was reverse transcribed with 20 μL of volume with Rs+dT primer (5'-GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
The PCR-amplified DNA fragments were purified using Qiagen columns and digested with EcoRI and HindIII 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.

**RESULTS**

**Isolation of LIP-36 from the Chloroplast Envelope of Low-CO\(\text{2}\)-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\(\text{2}\)-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\(\text{2}\) growth conditions. It is also very clear that LIP-36 is present in the chloroplast envelopes of low-CO\(\text{2}\)-grown cells, whereas it is missing in the chloroplast envelopes of high-CO\(\text{2}\)-grown cells (Fig. 1B). The chloroplast envelopes isolated from high- and low-CO\(\text{2}\)-grown cells were resolved on SDS-PAGE and transferred to Immobilon. The membrane was stained with Coomassie blue. Fifty micrograms of chloroplast envelope of low-CO\(\text{2}\)-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\(\text{2}\)-grown cells, stained with Coomassie blue. Fifty micrograms of chloroplast envelope from high (lane 1) and low CO\(\text{2}\) (lane 2) was loaded onto the gel. The arrow indicates LIP-36, which is present only in the chloroplast envelope of low-CO\(\text{2}\)-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 insert (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-CO₂-inducible clones, 417, 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

Gene Maps of LIP-36

![Gene Map](attachment:GeneMap.png)

**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.
Cloning of Two cDNAs Encoding LIP-36 Protein from *Chlamydomonas reinhardtii*

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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**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.

**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.
The hydropathy profile of the deduced amino acid sequence of LIP-36 GI. 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]-[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-CO₂ Growth Conditions**

The presence of two genes encoding the same protein, LIP-36, suggests that they may be regulated differently by CO₂. To determine whether this is the case, total RNA was isolated from CO₂-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-CO₂-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-CO₂ growth conditions. In contrast, the steady-state levels of both mRNAs dramatically increased and remained high after cells were switched from high-CO₂ to low-CO₂ 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 CO₂ 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-CO₂ 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-CO₂-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 regions 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-CO₂ growth conditions. The high-CO₂-grown cells were switched to low-CO₂ conditions at 0 h. Total RNA was isolated at 0, 4, and 10 h from cells left growing under high-CO₂ conditions (lanes C). Total RNA was also isolated from low-CO₂-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.
Cloning of Two cDNAs Encoding LIP-36 Protein from *Chlamydomonas reinhardtii*

**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, 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 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-oxygenolate/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-oxygenolate/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 transportor. 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 (Flugge et al., 1989), a Ca2+-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-oxygenolate/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 specifics 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).

**ACKNOWLEDGMENTS**

The authors thank Anne Geraghty and Martin Spalding for the antibody against LIP-36, and Dr. Richard Cook for his help in the amino acid sequencing.

Received November 4, 1996; accepted January 28, 1997.

Copyright Clearance Center: 0032-0889/97/114/0265/09.

The GenBank accession numbers for the DNA sequences reported in this article are U75345 (LIP-36 G1) and U75346 (LIP-36 G2).

**LITERATURE CITED**


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