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A novel α-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO_2

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A 29.5 kDa intracellular α-type carbonic anhydrase, designated Cah3, from the unicellular green alga *Chlamydomonas reinhardtii* is the first of this type discovered inside a photosynthetic eukaryote cell. We describe the cloning of a cDNA which encodes the protein. Immunoblot studies with specific antibodies raised against Cah3 demonstrate that the polypeptide is associated exclusively with the thylakoid membrane. The putative transit peptide suggests that Cah3 is directed to the thylakoid lumen, which is confirmed further by the presence of mature sized Cah3 after thermolysin treatment of intact thylakoids. Complementation of the high inorganic carbon concentration-requiring mutant, *cia-3*, with a subcloned cosmid containing the *cah3* gene yielded transformants that grew on atmospheric levels of CO_2 (0.035%) and contained an active 29.5 kDa α-type carbonic anhydrase. Although, *cia-3* has reduced internal carbonic anhydrase activity, unexpectedly the level of Cah3 was similar to that of the wild-type, suggesting that the mutant accumulates an inactive Cah3 polypeptide. Genomic sequence analysis of the mutant revealed two amino acid changes in the transit peptide. Results from photosynthesis and chlorophyll *a* fluorescence parameter measurements show that the *cia-3* mutant is photosynthetically impaired. Our results indicate that the carbonic anhydrase, extrinsically located within the chloroplast thylakoid lumen, is essential for growth of *C.reinhardtii* at ambient levels of CO_2, and that at these CO_2 concentrations the enzyme is required for optimal photosystem II photochemistry.

Keywords: CCM/*cia-3*/complementation/thylakoid lumen/transfer peptide

Introduction

CO_2 and HCO_3^- are substrates and products of many different metabolic reactions in cells. The uncatalysed interconversion between CO_2 and HCO_3^- is slow, with a $k_{1/2}$ for the hydration reaction of 14.1 s (Smith, 1988). Carbonic anhydrase (CA; carbonate dehydratase, carbonate hydrolyase, EC 4.2.1.1) catalyses the reversible reaction $CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$ with a maximum turnover number in excess of 10^6/s (Khalifah, 1971). To date, three phylogenetically independent gene families encoding distinct types of CAs have been identified (α-, β- and γ-CA; reviewed by Hewett-Emmett and Tashian, 1996) that share no significant sequence homology. CAs are involved in a variety of biological processes including pH regulation, respiration and photosynthesis (Tashian, 1989; Badger and Price, 1994).

Most is known about the α- and β- types, whereas the γ-type CA has been identified only recently in archaebacteria. The α-CA is the only type found in animals, but it also occurs in the periplasmic space of the eukaryotic unicellular green alga *Chlamydomonas reinhardtii* and in the eubacteria *Neisseria gonnorhoeae*. The β-CAs were first discovered in the stroma of higher plant chloroplasts, but have since been found in eubacteria (Hewett-Emmett and Tashian, 1996) and in algal mitochondria (Eriksson *et al*., 1996).

Aquatic plants must overcome the slow diffusion of CO_2 in water to photosynthesize effectively. The green alga, *C.reinhardtii*, and many other green algae and cyanobacteria have the ability to concentrate inorganic carbon (C_i) in response to varying external levels during growth. When cells are exposed to low C_i levels (in solutions equilibrated with ambient air containing 0.035% CO_2, for instance), a C_i-concentrating mechanism (CCM), is induced to actively supply CO_2 to Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39; Aizawa and Miyachi, 1986; Badger and Price, 1994). The synthesis of at least seven polypeptides is induced concomitantly with the CCM (Moroney *et al*., 1985; Fukuzawa *et al*., 1990; Rawat and Moroney, 1991), including a 37 kDa periplasmic α-CA (pCA, Fukuzawa *et al*., 1990), a 36 kDa chloroplast envelope polypeptide (Ramazanov *et al*., 1993), a 53 kDa polypeptide identified as an alanine-aminotransferase (Chen *et al*., 1996) and, recently, a 21 kDa mitochondrial β-CA (mtCA, Eriksson *et al*., 1996). The specific function of every one of the seven inducible polypeptides is so far unknown, although pCA is proposed to accelerate the equilibration of the predominant C_i species, HCO_3^- and CO_2, outside the plasma membrane (Moroney *et al*., 1985), thereby allowing CO_2 to enter the cell at a rate sufficient to support photosynthesis.

It has been inferred that CAs are also present within algal cells, as they are in higher plant cells, in the chloroplast and/or the cytoplasm (Spalding *et al*., 1983; Moroney and Mason, 1991; Sültemeyer *et al*., 1995; Amoroso *et al*., 1996). CA activity has also been found associated with, or in components tightly bound to, thylakoid membrane preparations from higher plants (Komarova *et al*., 1982) and green algae (Pronina and Semenenko, 1988; Pronina and Borodin, 1993). This activity is thought to be involved either in the regulation

of photosynthetic electron transport (Moubarak-Milad and Stemler, 1994) or as an important component of the CCM (Raven, 1997). We recently reported the purification of a 29.5 kDa intracellular α-CA from *C.reinhardtii* which is distinct from the pCA (Karlsson *et al*., 1995). A number of mutants have also been isolated that apparently lack an internal CA and require elevated levels of CO_2 for growth (Spalding *et al*., 1983; Moroney *et al*., 1986). One of these mutants, *cia-3*, fails to label a protein with a CA-specific photoaffinity label (Husic and Marcus, 1994) which in wild-type cells reacts with a 30 kDa protein.

Here we report the identification of an intracellular α-type CA exclusively associated with the thylakoid membrane and targeted to the chloroplast lumen. We describe the cloning of a full-length cDNA clone, designated *cah3*, encoding this protein. We also show that expression of the *cah3* gene can complement the phenotype of the *cia-3* mutant, restoring its intracellular CA activity and photosynthetic capacity, thereby allowing the transformants to grow in low CO_2 environments. We believe these results are of fundamental importance to the study of the CCM in algae and of photosynthesis in general, because they describe for the first time molecular evidence for a thylakoid-associated CA.

Results

Isolation and sequence of cah3 cDNA

Tryptic fragments obtained after cleavage of an intracellular 29.5 kDa CA polypeptide previously isolated from *C.reinhardtii* (Karlsson *et al*., 1995) were used to design degenerate oligonucleotide primers. A probe was obtained from a cDNA library, via PCR, and that was used to screen the same library for full-length cDNA clones. Several positive clones were identified and sequenced. The longest of these cDNA clones was 1383 bp and contained an open reading frame encoding a polypeptide of 310 amino acids (Figure 1A). The cDNA also had a 61 nucleotide 5′-untranslated region and a 389 nucleotide 3′-untranslated region (excluding the polyadenylated tail). A polyadenylation signal (TGTAA) characteristic of nuclear-encoded genes in *C.reinhardtii* (Silflow *et al*., 1985) was located 372 bp downstream of the stop codon (Figure 1A). Subsequent genomic Southern hybridization indicated that *cah3* is a single copy gene (results not shown). The calculated molecular mass of the mature polypeptide is 26 058 Da, with a predicted isoelectric point of 7.87. The putative start codon is 72 amino acids upstream of the mature N-terminus, as previously determined by direct sequencing of isolated Cah3 protein (Karlsson *et al*., 1995). When the deduced amino acid sequence of the mature Cah3 polypeptide was compared with other known CAs, the highest degree of similarity was found to those of the α-type (Figure 1B). Overall, Cah3 shares 30–40% identity with other α-CAs, with up to 90% identity within the conserved domains characteristic of this CA type. It is most similar to the α-CA from the bacterium, *N.gonorrhoeae*, with which it shares 40.6% identity. Within the conserved domains, Cah3 possesses 26 of the 36 amino acid residues with side chains that project into or border the active site cavity in mammalian α-CAs (Hewett-Emmett and Tashian, 1996; Figure 1A). Furthermore, the three histidine residues that function as Zn ligands within the active site of all α-CAs are also present in Cah3 (Figure 1A).

The 72 amino acid pre-sequence of Cah3 contains sequence elements that are characteristic of proteins targeted into the thylakoid membrane lumen. The lumen-targeting transit peptides in *C.reinhardtii* are bipartites of an N-terminal stroma-targeting domain and a C-terminal lumen-targeting domain (Franzén *et al*., 1990). The stromal-targeting domain is cleaved by a stromal peptidase at a semiconserved cleavage site, Val-X-Ala, proposed to be Val-Arg-Ala in Cah3 (Figure 1C). The lumen-targeting domain contains a hydrophobic region followed by a conserved lumen peptidase cleavage site, Ala-X-Ala. In Cah3, the hydrophobic region is followed by Ala-Lys-Ala (Figure 1C).

In higher plants, some polypeptides targeted to the lumen are translocated by a ΔpH-dependent process, which is distinct from the Sec-related processes. Figure 1C shows a comparison of the transit peptides of Cah3 from wild-type *C.reinhardtii* and the *cia-3* mutant, together with the *C.reinhardtii* oxygen-evolving enhancer proteins, OEE2 and OEE3, the barley photosystem I reaction centre subunit psaN (PSI-N) and the tomato polyphenol oxidase A (PPO) transfer peptides. The corresponding higher plant OEE2 and OEE3 polypeptides from wheat (23 kDa) and spinach (16 kDa) respectively, and the barley PSI-N and tomato PPO have all been shown to be imported by the ΔpH-dependent translocation pathway (Nielsen *et al*., 1994; Chaddock *et al*., 1995). A comparison of these higher plant transfer peptides shows a high degree of similarity, with a characteristic twin arginine motif in the lumen-targeting domain, closely followed by the hydrophobic region (Chaddock *et al*., 1995). It is therefore plausible that the *C.reinhardtii* OEE2, OEE3 and Cah3 polypeptides are translocated via the same trans-thylakoid proton gradient mechanism, consistent with the hypothesis that Cah3 is directed to the thylakoid lumen. This hypothesis is strengthened further by the presence of a lysine residue in the C-terminus of Cah3, a few residues before the conserved lumen peptidase cleavage site, which recently has been shown to be an important part of a 'Sec-avoidance' signal (Bogsch *et al*., 1997).

These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under Accession number U40871.

Regulation of cah3 by external CO_2 concentration during growth

Since intracellular CA has been proposed to be involved in the CCM (Spalding *et al*., 1983), the expression of the *cah3* gene in wild-type (wt-*137c*) *C.reinhardtii* was measured upon changing CO_2 levels from 5% (high C_i) to atmospheric levels (low C_i; i.e. 0.035% CO_2) during cultivation. Northern blot hybridization of total RNA isolated from high and low C_i-grown cells (Figure 2) demonstrated that the *cah3* gene is expressed under high C_i as a transcript of 1.45 kb. The level of this transcript, however, approximately doubles after transfer of the culture to low C_i relative to the amount of 16S/18S rRNA present. Induction of the CCM by this low C_i treatment was confirmed by analysing the induction of the low C_i-inducible 21 kDa CA located in the mitochondria (Eriksson *et al*., 1996); no transcripts for this gene were detected

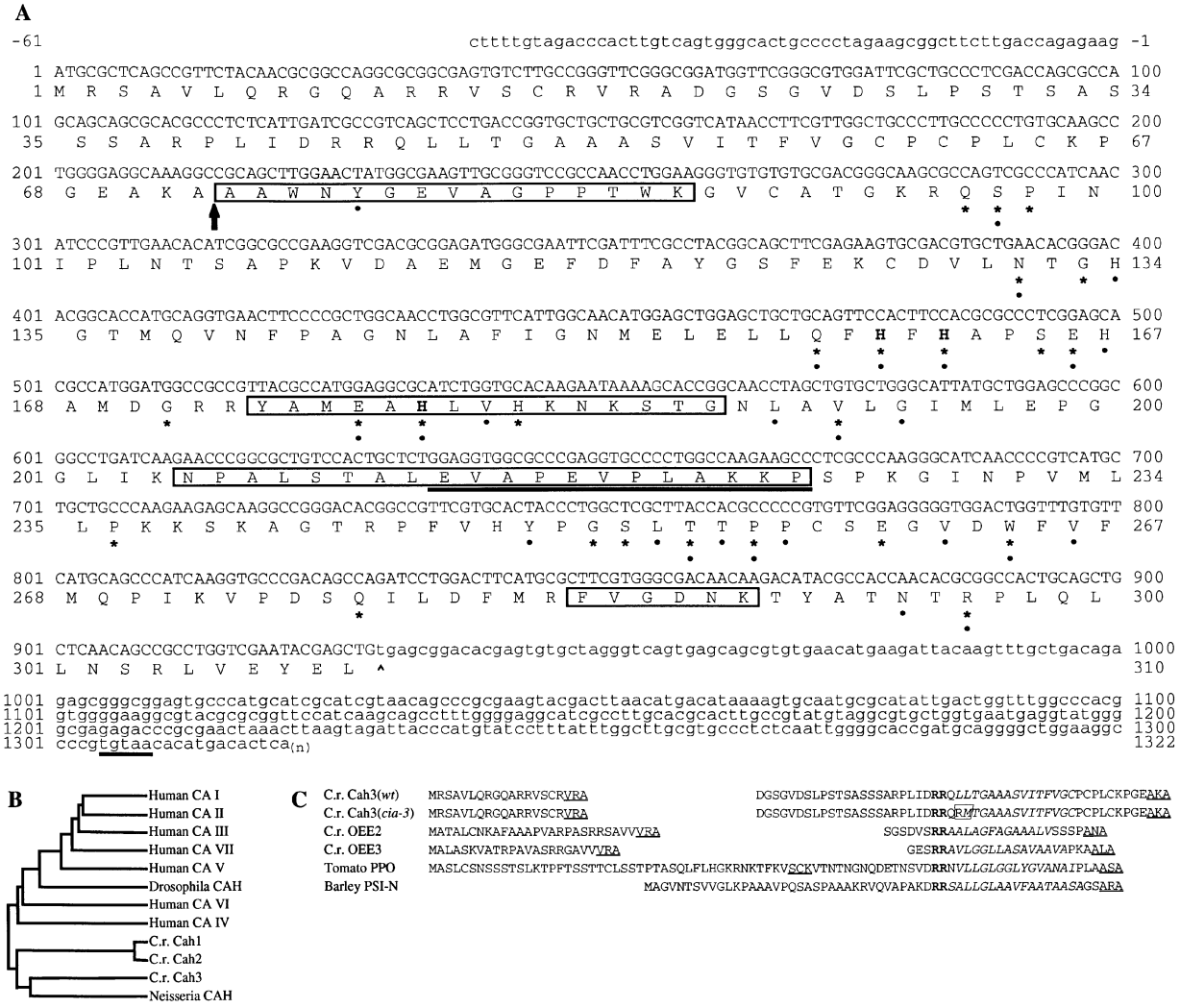

Fig. 1. Sequence of *cah3* from *C.reinhardtii*, schematic relationship to other CAs and transit peptide alignment. (**A**) The sequence of the cloned *cah3* cDNA from *C.reinhardtii* and the deduced amino acid sequence of Cah3. The arrow indicates the cleavage site between the putative transit peptide and the mature polypeptide. Boxed amino acids correspond to sequences obtained from tryptic fragments of the mature protein and N-terminus determination. The underlined amino acid sequence was used for custom antibody production. Underlined nucleotides in the 3′-untranslated region indicate a signal for polyadenylation. Conserved amino acids, forming the active site, are denoted by ●, while the * indicates completely conserved amino acids among the CAs below. The three conserved zinc-liganded histidine residues at the active site are shown in bold. (**B**) A dendogram of the pairwise relationships between the *C.reinhardtii* Cah3 (U40871), Cah1 (D90206) and Cah2 (D90207), human CAI (M33987), CAII (M77181), CAIII (M29458), CAIV (L10955), CAV (L19297), CAVI (M57892) and CAVII (M76423), *Drosophila melanogaster* CAH (L39622) and *Neisseria gonorrhoeae* CAH (ORF2, U11547). The *D.melanogaster* CAH was obtained by removing a single intron (1227–1294, 67 bp) from L39622 as described in Hewett-Emmett and Tashian, 1996). The relationships were established using the GCG (Genetics Computer Group, Madison, WI) PILEUP program. (**C**) A comparison of the transit peptides from the wild-type Cah3 and the *cia-3* mutant Cah3 together with the *C.reinhardtii* oxygen-evolving enhancer proteins, OEE2 and OEE3, barley PSI reaction centre subunit psaN (PSI-N) and the tomato polyphenol oxidase A (PPO), transit peptides. The N-terminal stromal and the C-terminal lumenal processing peptidase cleavage sites are underlined, and the twin arginine motif in each sequence is showed in bold. The hydrophobic region is shown in italics. The two amino acid changes in the mutant Cah3 are boxed.

under high C_i but strong induction occurred after transfer to low C_i, indicating that the changed conditions had induced CCM.

Localization of Cah3 by immunoblot analysis

To examine the localization of Cah3 within the cell, specific antisera were raised against a synthetic peptide derived from an internal sequence of the *cah3* gene product. As shown in Figure 3, the antibodies recognized a polypeptide of ~30 kDa in isolated chloroplasts from high and low C_i-grown *C.reinhardtii* cells (lanes 3 and 4). Cah3 was located only in the chloroplast thylakoid fractions (lanes 7 and 8) and not in the stromal fraction (data not shown), nor in the fraction containing chloroplast envelope membrane proteins (lanes 5 and 6). Differences were not apparent in the amount of Cah3 protein in high and low C_i-grown cells, in contrast to the slight induction of *cah3* transcripts observed following the shift to low C_i conditions.

Identification of Cah3 in the CCM mutant, cia-3

Several mutants of *C.reinhardtii* have been isolated that are apparently deficient in intracellular CA activity (Spalding *et al.*, 1983; Moroney *et al.*, 1986; Katzman *et al.*, 1994). Previously, we described an active site-directed photoaffinity reagent, [^{125}I]*p*-aminomethylbenzene-sulfonamide-4-azidosalicylamide ([^{125}I]PAMBS-ASA), that upon photoactivation specifically covalently modifies

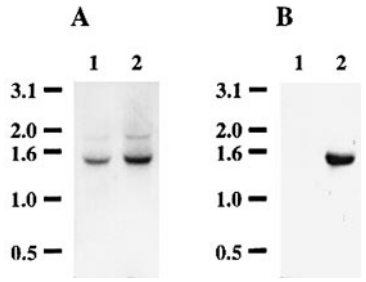

Fig. 2. Northern hybridization analyses of wild-type (wt-*137c*) cells grown in high and low C_i. Comparison of *C.reinhardtii* total RNA (10 μg) isolated from high C_i- (lane 1) and low C_i- grown cells (lane 2). Filter A was hybridized with the *cah3*-specific probe. Filter B was hybridized with the low C_i-inducible mitochondrial 21 kDa CA-specific probe. Denatured DNA size standards in kb (Gibco-BRL) are indicated on the left.

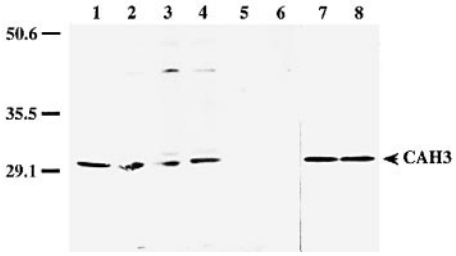

Fig. 3. Immunoblot analysis of whole cell and chloroplast fractions. A comparison of whole cells (lanes 1 and 2), chloroplasts (lanes 3 and 4), envelope membranes (lanes 5 and 6) and thylakoid membranes (lanes 7 and 8). High C_i-grown cells and subcellular fractions were used for lanes 1, 3, 5 and 7. Low C_i cells and subcellular fractions were used for lanes 2, 4, 6 and 8. 150 μg of protein was loaded in whole cell lanes and 15 μg in the subcellular fraction lanes. Cah3-specific, custom-made antibodies were used. Molecular mass markers are shown on the left.

and labels CA. Using this reagent, a 30 kDa polypeptide believed to be the chloroplastic CA was labelled in wild-type (wt-*137c*) cells but not in the CA-deficient mutant *cia-3* (Husic and Marcus, 1994). We therefore transformed the double mutant strain *cia-3/cw-15* with a cosmid (~40 kbp of genomic DNA) containing the *cah3* gene and selected for cells that could grow on low C_i. Approximately 45 transformants were recovered from two separate transformations. In contrast to the original strain, most of these transformants could grow well on ambient CO_2 (Figure 4). A subcloned 4 kbp piece of the genomic clone containing the *cah3* gene was also able to complement the *cia-3* phenotype, minimizing the possibility that another gene present on the large cosmid was responsible for the recovery of *cia-3*.

Following photoaffinity labelling, the 30 kDa polypeptide labelled in wild-type cells was observed in the transformants, but not in the original *cia-3* or *cia-3*/*cw-15* mutants (Figure 5). Furthermore, when the *cia-3* strain was crossed with the wild-type, analysis of the resultant tetrad progeny (Table I; TD1–TD4) revealed co-segregation of the presence of the 30 kDa photoaffinity-labelled polypeptide and the efficient C_i utilization characteristic of wild-type cells. These results are consistent with there being a link between the loss of a putative internal CA and the growth requirement for high C_i. This indicates that the transformants express the gene encoding an active internal CA which enables them to grow on low C_i.

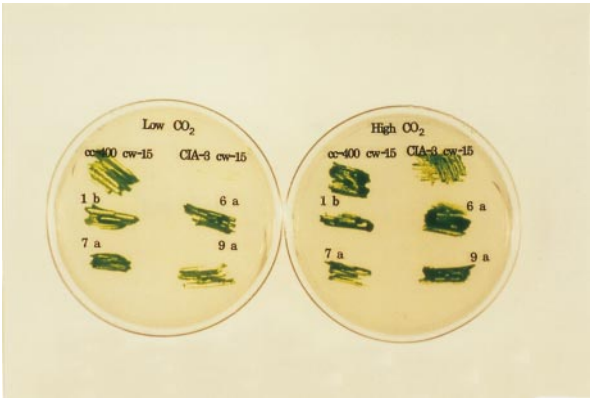

Fig. 4. Growth of double mutant strain *cia-3*/*cw-15* and strains transformed with cosmids containing the *cah3* gene. Strains *cc-400*, *cia-3*/*cw-15* and four independent transformants (1b, 6a, 7a and 9a) were plated on minimal media and grown for 7 days under elevated CO_2 conditions (High CO_2) or under ambient air (Low CO_2).

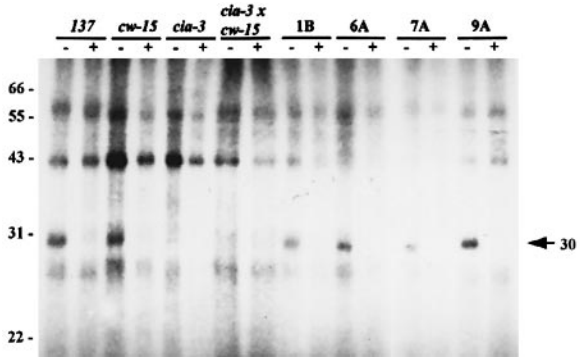

Fig. 5. Photoaffinity labelling of *cia-3*/*cw-15* cells transformed with the *cah3* gene. Extracts from the indicated cell lines were labelled with the CA-directed photoaffinity label [^{125}I]PAMBS-ASA as in Husic and Marcus (1994). Specific labelling of the 30 kDa polypeptide (marked with an arrow) is judged by those proteins labelled in the absence (–) but not in the presence (+) of an excess of an unlabelled competing sulfonamide, ethoxyzolamide (Husic and Marcus, 1994). Samples were wild-type cells (wt-*137c*), the cell wall-deficient mutant *cw-15* (cc400), the CA-deficient mutant *cia-3*, a *cia-3*/*cw-15* double mutant, and four strains of *cia-3*/*cw-15* transformed with the *cah3* gene that were capable of growth on atmospheric levels of CO_2 (1B, 6A, 7A and 9A). Molecular mass markers are shown on the left.

Table I. Characterization of tetrad progeny (TD1–TD4) of a cross between the CCM mutant (*cia-3*) and wild-type (*cc-124*) *C.reinhardtii* cells

Cell line	$K_{0.5}CO_2$ (μM)	Photoaffinity-labelled 30 kDa	Immunoreactive 30 kDa
cc-124	3	+	+
cia-3	45	–	+
TD1	7	+	+
TD2	26	–	+
TD3	2.5	+	+
TD4	37	–	+

Chlamydomonas reinhardtii cells were grown at 5% CO_2, and transferred to atmospheric levels of CO_2, 5 h before harvesting. Photosynthesis rates at saturating levels of CO_2 were >140 μmol/h/mg Chl for all strains. The presence of the 30 kDa polypeptide was determined by photoaffinity labelling of cell extracts with [^{125}I]PAMBS-ASA and by immunoblot analyses with Cah3-specific antibodies.

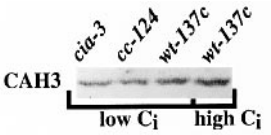

Fig. 6. Immunoblot analysis of mutant and wild-type strains. A comparison of low C_i-grown mutant strain *cia-3*, low C_i-grown wild-type strains *cc-124* (mt–) and *137c* (mt+) and high C_i-grown *137c*. Whole cell protein (150 μg) was loaded in each lane, and the Cah3 protein was detected using specific antibodies raised against Cah3.

Moreover, internal CA activities measured in the transformants were found to be in the same range as that of the wild-type (80–120%).

Immunoblot analyses of total protein extracts from the *cia-3* strain with the Cah3-specific antisera indicated, surprisingly, that the mutant had a level of Cah3 similar to the wild-type (Figure 6). This strongly suggests that the phenotype of this CCM mutant results from the production of an inactive Cah3 protein rather than the inactivation of Cah3 synthesis *per se*.

Sequencing of the genomic region of the *cah3* gene in the *cia-3* mutant revealed two point mutations in the transit peptide. These mutations, T→G (position137; Figure 1A) and C→A (position 139), causes changes in the amino acid composition, with a leucine pair becoming arginine and methionine. As shown in Figure 1C, these changes occur at the beginning of the hydrophobic region close to the twin arginines that are known to be important for the ΔpH-dependent thylakoid protein translocase (Chaddock *et al*., 1995).

The thermolysin treatment of thylakoids from wild-type and mutant thylakoids analysed by immunoblot with specific antibodies (Figure 7A) shows that the mutant Cah3 is digested both in intact and sonicated thylakoids (lanes 4 and 6) while the wild-type protein is only degraded in the sonicated sample (lanes 3 and 5). The extrinsic oxygen-evolving enhancer protein OEE2, probed with a higher plant OEC-24 antibody, is used as a control (Figure 7B). The control lanes 7 and 8, where EDTA is added before thermolysin, shows that no unspecific digestion is occurring.

Impaired photosynthetic capacity of the CCM mutant, cia-3

The mutant *cia-3* has a severely impaired photosynthetic capacity under low C_i conditions as determined by photosynthetic and chlorophyll *a* fluorescence measurements. Figure 8A shows the photosynthetic capacity as a function of the amount of bicarbonate added for high C_i- and 24 h low C_i-adapted wild-type and mutant cells. The high C_i cells were very similar with respect to their C_i dependency. However, while the low C_i-grown wild-type cells exhibited characteristically high C_i affinity, the low C_i-grown *cia-3* cells had low affinity, similar to the affinity of cells grown in high C_i. The maximum photosynthetic capacity of these cells was also lower, reaching only about half the photosynthetic rate of the other cell types. Figure 8B shows the C_i-dependent response of the reduction of the primary electron acceptor (Q_A), as estimated by 1 – F/Fm′ (Genty *et al*., 1989). This parameter is indicative of photosystem II (PSII) efficiency, and it clearly shows that

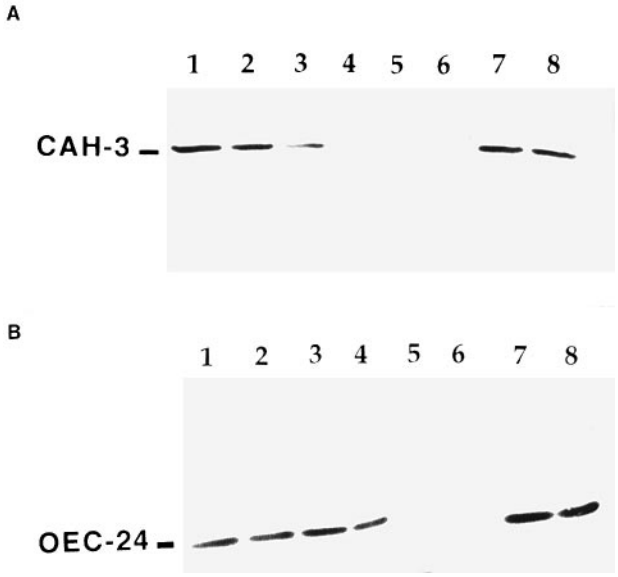

Fig. 7. Thermolysin treatment of thylakoid membranes prepared from Cah3 wild-type (*cc400*; lanes 1, 3, 5 and 7) and Cah3 mutant (*cia-3*/*cw-15*; lanes 2, 4, 6 and 8). (**A**) Immunoblot analyses of samples with Cah3-specific antibodies and (**B**) immunoblot with OEE2-specific antibodies (raised against pea OEC-24). Lanes 1 and 2 are untreated samples, lanes 3–8 were treated with thermolysin at a concentration of 75 μg/ml and a chlorophyll concentration of 250 μg/ml. Thylakoids in lanes 5 and 6 were disrupted by sonication before treatment. In lanes 3–6, the reactions were stopped with the addition of 10 mM EDTA after incubation. In the control lanes 7–8, EDTA was added before the addition of thermolysin.

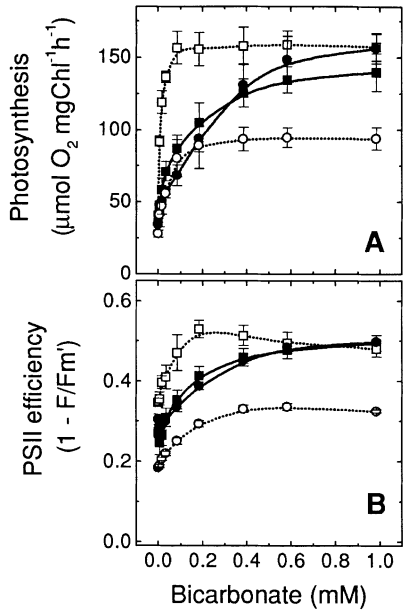

Fig. 8. Photosynthetic characteristics of wild-type and the *cia-3* mutant strain. Changes in the rate of photosynthetic O_2 evolution (**A**) and the photochemical efficiency of PSII (**B**) as a function of bicarbonate concentration in wild-type (■ and □) and *cia-3* cells (● and ○). Cells grown in 5% CO_2 (■ and ●) or adapted to ambient air for 24 h (□ and ○) were washed three times with CO_2-free growth medium, prior to measurements.

the low C_i-grown mutant cells suffer from a severe decrease in PSII efficiency compared with the wild-type. Even under higher bicarbonate concentrations, the mutant did not recover PSII efficiency or normal rates of photo-

synthesis. The reduction of photosynthetic capacity of the mutant after the transfer from high to low C_i conditions also increased with time (data not shown). Photosynthetic rates in the mutant dropped after 2–4 h at low C_i, and had decreased by 35–40% after 24 h.

Discussion

Cah3 is an α-type intracellular carbonic anhydrase in Chlamydomonas reinhardtii

We have described in this study the cloning and sequencing of a gene which encodes an intracellular CA of the α-type from *C.reinhardtii*. The deduced amino acid sequence of a full-length cDNA contained both the tryptic fragments and the N-terminus reported earlier (Karlsson *et al*., 1995). The predicted Cah3 polypeptide is the first α-type CA to be found within a photosynthetic eukaryotic cell. Although it has relatively low sequence similarity to animal α-type CAs (40% identity), the algal homologue has most of the conserved domains characteristic of this CA family. Most prominent are the three histidine residues that act as ligands for the Zn atom in the active site of the protein; residues 160, 162 and 179 in Cah3 (Figure 1A). The CA activity of the purified enzyme, measured by Karlsson *et al*. (1995), also confirms that this is a CA.

Chloroplastic localization of Cah3

Using specific antibodies raised against the 29.5 kDa CA protein, we have shown that Cah3 is located in the chloroplast, where it is localized exclusively in the thylakoid membrane, and is not associated with the chloroplast envelope (Figure 3). Previous mass spectrometric measurements on intact cells and isolated organelles led Amoroso *et al*. (1996) to suggest the presence of three intracellular CAs, two of which were believed to be associated with the chloroplast. One of the chloroplast forms was proposed to be a soluble CA while the other was believed to be membrane-associated. In our earlier work (Karlsson *et al*., 1995), however, we found evidence for only one intracellular, membrane-associated CA activity. In this study, using specific Cah3 antisera, we found no evidence for more than one α-type CA. This of course does not exclude the possibility that other types of CA (β or γ) may exist in the chloroplast of *C.reinhardtii*.

Sequence analyses of the Cah3 (Figure 1C) pre-sequence reveal striking similarities with pre-sequences known to specify translocation of proteins into the thylakoid lumen. The sequence also fulfils the criteria for transfer peptides translocated via the ΔpH-dependent thylakoidal protein translocase; the characteristic twin arginine motif and a C-terminal lysine residue (Chaddock *et al*., 1995; Bogsch *et al*., 1997; reviewed by Robinson and Mant, 1997). The sequence information together with Western blot data (Figure 3) supports our belief that Cah3 is a protein located in the thylakoid lumen.

All immunological data were confirmed using antibodies raised against a Cah3 polypeptide produced in *Escherichia coli* by overexpression of the region of the *cah3* gene encoding the mature Cah3 polypeptide (data not shown).

Expression of cah3 increases when the external CO_2 level decreases

There was an approximate doubling of *cah3* mRNA in relation to the amount of rRNA present when cells were transferred from high to low C_i conditions (Figure 2). This level of induction is consistent with that proposed for the three intracellular CAs apparently induced under these conditions—which are estimated to increase 2-(Amorosos *et al*., 1996) to 10-fold (Sültemeyer *et al*., 1995). A corresponding rise in CA protein content was not apparent on immunoblots, although it is possible that post-transcriptional and/or -translational regulation occurs to maintain a steady-state level of this CA in *C.reinhardtii*.

Inactive intracellular CA is present in the high C_i-requiring mutant, cia-3

Immunological examination of total cellular protein extract from the high C_i-requiring mutant, *cia-3*, indicated that it contained amounts of Cah3 similar to that of the wild-type (Figure 6). However, the CA-directed photoaffinity labelling showed that a 30 kDa polypeptide was labelled in wild-type cell extracts but not in the *cia-3* mutant extracts. Although the obvious explanation was that *cia-3* lacked this 30 kDa polypeptide, the CA identified by Husic and Marcus (1994) and the CA we have isolated previously (Karlsson *et al*., 1995) are very probably the same intracellular CA, since complementation of the mutant with the *cah3* gene resulted in cell lines which produced the photoaffinity-labelled protein (Figure 5). The transformants also regained intracellular CA activity.

This hypothesis was strengthened further by crossing *cia-3* with wild-type cells. Two of the resultant tetrad progeny (TD1 and TD3) showed mutant characteristics; a low efficiency for C_i utilization coupled with the absence of the 30 kDa labelled polypeptide (Table I), whereas the other two progeny (TD2 and TD4) showed both the presence of the 30 kDa photoaffinity-labelled polypeptide and the ability to grow on low CO_2, characteristic of wild-type cells. Since all progeny showed cross-reaction of a 30 kDa polypeptide with the Cah3-specific antibodies, it is therefore unlikely that *cia-3* lacks this polypeptide.

We next considered whether the activity of the chloroplastic CA in *cia-3* had been impaired by a mutation which caused a loss of the binding of the sulfonamide photoaffinity label inhibitor. Sulfonamides have been shown to bind to the active site cavity of an α-CA (Liljas *et al*., 1994). Unexpectedly, sequencing of the *cah3* genomic DNA from *cia-3* did not reveal a mutation in the mature part of the protein, but instead revealed two point mutations in the sequence encoding the transfer peptide (Figure 1C), leading to changes in the amino acid composition there; an arginine and methionine for two leucines. These changes occur one amino acid after the twin arginine motif, which is known to be important for the trans-thylakoid proton gradient transport mechanism (Chaddock *et al*., 1995). Thus, the inactivation of the Cah3 protein is not caused by a mutation within the mature enzyme, but by sequence changes in the transfer peptide, which may lead to mistargeting, misfolding or incorrect cleavage of the Cah3 precursor. A mistargeting of the Cah3 polypeptide could in turn lead to improper folding and/or diminished activity because of the altered environment. In fact, the digestion of the mutant Cah3 in intact thylakoids (Figure 7) indicates that this protein is accessible for proteolytic attack while the wild-type protein is not. This strengthens both the assumption that the mutant has an impaired import of the Cah3 precursor into

the lumen and that the wild-type mature Cah3 is located extrinsically in the thylakoid lumen. The wild-type pattern equals that of the control protein, OEE2.

Expression of the *cah3* gene in the *cia-3* mutant was confirmed by Northern hybridization (data not shown). The fact that the mutant expresses full-length transcripts, although at low levels, supports the conclusion suggested by the sequence data that a mutation in the transfer peptide is responsible for the inactivation of the *cia-3* CA.

Function of thylakoid lumen CA

After exposure to low C_i conditions for 24 h, the high C_i-requiring mutant, *cia-3*, suffers severely from low PSII efficiency and cannot sustain positive net photosynthesis during non-saturating light conditions, as shown by the results in Figure 8. It was reported earlier that this mutant accumulates C_i inside the cell (Moroney *et al*., 1986) but still does not grow at ambient levels of CO_2. Our discovery of a lumen-targeted CA is the experimental support for models inferring the presence of a CA within the lumen. Pronina and Semenenko (1990) and Pronina and Borodin (1993) have proposed a model in which a hypothetical membrane-associated CA in the lumen dehydrates HCO_3^- taken up from the stroma in parallel with the active light-driven H^+ influx. In the light, CO_2 is the major C_i form at equilibrium in the acidic lumen, and this could then passively diffuse through the thylakoid membrane to a carboxylating site in the stroma close to the outer thylakoid membrane surface (Pronina and Semenenko, 1990) raising the effective CO_2 concentration available to Rubisco. This model with a lumenal CA has been developed further and analysed quantitatively by Raven (1997) and found to be qualitatively plausible. Our results from the mutant work support Raven's model in that the mutant still accumulates C_i, but in the high pH of the stroma in light, it occurs predominantly as HCO_3^- and thus cannot act as substrate for Rubisco. The uncatalysed conversion to CO_2 in the stroma is also too slow to support high rates of photosynthesis. According to the model, HCO_3^- is taken up into the lumen but in the mutant the catalysed conversion of HCO_3^- to CO_2 does not take place, thus Rubisco cannot be supported fully with substrate via this pathway either. This would lead to a lowered CO_2 fixation rate, a lowered Calvin cycle turnover and, in turn, to a down-regulation of PSII efficiency (Baker and Boyer, 1994), as seen in Figure 8.

However, it has also been suggested that CA is associated with the photosynthetic electron transport chain close to PSII since certain inhibitors of CA also inhibit PSII activity (Stemler and Jursinic, 1983; Stemler, 1985). The mechanism for the 'bicarbonate effect' on PSII, discovered by Warburg and Krippahl (1960) and reviewed by Govindjee (1995), is still not elucidated fully. It has been proposed that a thylakoid CA might be involved in this bicarbonate effect (Moubarak-Milad and Stemler, 1994), ensuring that HCO_3^- ions are available to stimulate effective transport of electrons through PSII. Although evidence for this proposal is so far preliminary, the identification of Cah3 as a thylakoid-associated CA allows us to test this hypothesis experimentally for the first time. We are now pursuing a more detailed examination of the specific role(s) of this CA and its specific location with respect to PSI and PSII centres in *C.reinhardtii* under both low and high C_i growth conditions.

Summary

In this report, we have shown by molecular complementation that the protein absent in strain *cia-3* is an active Cah3. In addition, only transformants expressing an active Cah3 can grow at ambient levels of CO_2. These results are consistent with the idea that the chloroplast thylakoid CA encoded by *cah3* is an essential constituent of the enzymatic system that regulates photosynthetic carbon assimilation in *C.reinhardtii*.

Materials and methods

Algal strains and culture conditions

Wild-type *C.reinhardtii 137c* (mt+) was obtained originally from Dr R.K.Togasaki, Indiana University, whereas strains *cc-124* and *cc-400* (*cw-15*) were obtained from the *Chlamydomonas* Culture Collection at Duke University. All strains were grown in batch cultures at 25°C under a continuous irradiance of 150 μmol photons/m^2/s. Cultures containing 2 l of minimal medium were bubbled vigorously with air containing 5% CO_2. These cells are referred to as high C_i-adapted cells lacking the CCM. Low C_i-adapted cells were obtained by equilibrating the culture with air (i.e. 0.035% CO_2) 6–24 h prior to harvest. The major constituents of the medium were prepared according to Sueoka (1960), and the trace element solution was prepared according to Hutner *et al*. (1950). Crosses of *cc-124* (mt–) and *cia-3* were completed and the resultant progeny were characterized phenotypically as previously described (Moroney *et al*., 1986).

Screening of cDNA library, cloning and sequencing

A cDNA library was constructed using the Stratagene Uni-ZAPII kit (Stratagene, La Jolla, CA) from 5 μg of mRNA isolated from *C.reinhardtii* cells adapted to low C_i conditions. Double-stranded cDNA was ligated into the λZAPII arms, packaged with the Gigapack Gold kit, and transfected into the *E.coli* strain SURE (Burow *et al*., 1996).

The *C.reinhardtii* 29.5 kDa intracellular CA was purified as described in Karlsson *et al*. (1995). Three partial amino acid sequences were determined after tryptic cleavage as described earlier (Karlsson *et al*., 1995; Figure 1A). From these sequences, two degenerate oligonucleotides were synthesized, taking into account known *C.reinhardtii* codon usage. Inosine was used to reduce the degeneracy of the primers, and *Eco*RI restriction sites were included at the 5′ ends to facilitate cloning. The primers were: p1, 5′-GAAGAATTCIATGGAGGCICA(C/T)CTIGT-ICA(C/T)AAG-3′; and p2, 5′-GTGGAATTCGAGGTICCICTIGCIAA-GAAGCC-3′. PCR was performed, using either p1 or p2 and the vector-specific T7 primer at an annealing temperature of 52°C for 1 min, using total DNA from the cDNA library as template, and *Taq* polymerase (Perkin-Elmer) for extension. The p1 and T7 primer combination gave an 850 bp product, and the p2 and T7 primer produced a 700 bp product. The p2/T7 product could also be obtained using the p1/T7 product as template, confirming the common origin of the two products. A 380 bp *Eco*RI–*Pst*I fragment of the p1/T7 product was cloned into pUC19 (Sambrook *et al*., 1989) and sequenced using a thermal cycle amplification system (fmol DNA Sequencing System, Promega, Madison, WI). The 380 bp fragment was radiolabelled and used to screen ~200 000 recombinant phages from the cDNA library. Several positive clones were selected and the cDNA-pBluescript plasmids were excised according to the manufacturer's instructions (Stratagene, La Jolla, CA). The cloned cDNAs were sequenced using the system described above.

Genomic clones were obtained by screening a cosmid library (Purton and Rochaix, 1994) with the radiolabelled *cah3* cDNA (Sambrook *et al*., 1989). The presence of the complete *cah3* gene in the selected cosmids (each containing ~40 kbp of genomic DNA) was verified by partial sequencing of the insert using primers designed from the cDNA sequence, and was confirmed to be identical to the genomic *cah3* sequence submitted by Funke *et al*. (1997; DDBJ/EMBL/GenBank Accession number U73856). The *C.reinhardtii* strain *cia-3*/*cw-15* (Moroney *et al*., 1986) was transformed using the glass bead method of Kindle (1990). In addition, a subcloned 4 kbp *Hin*dIII–*Kpn*I fragment of the cosmid, containing the *cah3* gene, was used for complementation, to verify that this gene alone was necessary for complementation.

Genomic clones of the *cah3* gene in the mutant *cia-3* were obtained by PCR using *cia-3* genomic DNA as template and primers designed using the wild-type *cah3* gene. The resulting 1760 bp fragment, containing the complete coding strand, was cloned into pUC19 (Sambrook *et al*., 1989). Eight isolated plasmids were sequenced and confirmed as being identical.

RNA blot hybridization

Total nucleic acids were isolated from algae grown in high and low C_i according to Johanningmeier and Howell (1984). RNA was purified using the RNeasy Total RNA Kit (Qiagen). Northern blot analysis of RNA (10 μg) denatured by glyoxylation was performed according to Sambrook *et al*. (1989). Transcripts of the *cah3* gene were detected using a ^{32}P-labelled 780 bp DNA fragment (Figure 1A; nucleotides 224–1005). A ^{32}P-labelled cDNA which encodes the low C_i-inducible mitochondrial CA (Eriksson *et al*., 1996) was used as a control for CCM induction. Hybridization and washing steps were done at 50°C according to Sambrook *et al*. (1989). The amount of specific mRNA in each sample was determined directly from the filter using a GS 250 Molecular Imager (Bio-Rad) and accompanying PhosphoAnalyst software, and quantified relative to the amount of 16S/18S rRNA as described in Kulharni *et al*. (1992).

Protein electrophoresis, cell fractionation, immunoblotting and photoaffinity labelling

For protein analyses, cells were harvested and washed once in a buffer containing 25 mM HEPES-KOH pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride and 0.1 mM dithiothreitol (DTT). Samples were resuspended in a buffer containing 20 mM bis-tris-propane pH 7.0, 5 mM $MgCl_2$, 5 mM DTT, 0.2 mM ATP, 10 μM leupeptin and 2 mM benzamidine, then frozen at –80° C until used for electrophoresis. Intact chloroplasts were isolated as described by Mason *et al*. (1991), and chloroplast envelope membranes as described by Ramazanov *et al*. (1993). Thylakoid membranes were isolated according to the method of Allen and Staehelin (1993). Proteins were separated on 8–15% gradient polyacrylamide gels (0.8% bisacrylamide) as described previously (Laemmli, 1970). Immunoblotting was performed as described in the protocol from Bio-Rad Laboratories. Antibodies directed against a synthetic peptide derived from an internal amino acid sequence of Cah3 (residues 213–224; Figure 1A) were obtained from Research Genetics (Huntsville, AL).

Chlamydomonas reinhardtii cell extracts for photoaffinity labelling experiments were prepared and were labelled with the CA-directed photoaffinity reagent [^{125}I]PAMBS-ASA, as described previously (Husic and Marcus, 1994).

Thermolysin treatments of isolated Cah3 wild-type (*cw15*) and Cah3 mutant (*cia3/cw15*) thylakoids were done as described previously (Brock *et al*., 1993). In the reactions, 75 μg/ml thermolysin was used for chlorophyll concentrations of 250 μg/ml. After the incubation, the reactions were stopped with the addition of EDTA. The samples were analysed after denaturing gel electrophoresis and immunoblotting as described above. The antibodies used were Cah3 and OEE2 specific (raised against pea OEC-24, originally from Dr R.Henry, University of Florida).

Photosynthesis and chlorophyll fluorescence parameters

Photosynthetic O_2 exchange rates at 400 μmol photons/m^2/s of white light were measured at 25°C using a Clark-type O_2 electrode (Hansatech, King's Lynn, UK) with cells suspended to 3 μg chlorophyll/ml in CO_2-free growth media. The reaction was initiated by adding various concentrations of $NaHCO_3$. During measurements of O_2 evolution, the chlorophyll fluorescence parameters, F (chlorophyll fluorescence yield during illumination) and Fm′ (chlorophyll fluorescence yield when all PSIIs are closed transiently by a saturating light pulse of 500 ms duration and 6000 μmol photons/m^2/s intensity) were measured simultaneously using a PAM chlorophyll fluorometer (Watz, Effeltrich, Germany), and the efficiency of reduction of primary quinone electron acceptor (Q_A) was estimated as (1 – F/Fm′) (Genty *et al*., 1989).

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