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Discovery of Novel Phosphoenolpyruvate Carboxylase (PEPC) Genes and Their Active Polypeptides in the Green Microalga *Chlamydomonas reinhardtii*

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This work describes the discovery of novel phosphoenolpyruvate carboxylase (PEPC) genes and their active catalitic polypeptides in the green microalga *Chlamydomonas reinhardtii*. Green-algal PEPC has been unexplored before in molecular terms. Our recent studies have reported the molecular cloning of the two PEPC genes, *Ppc* genes in *C.reinhardtii* (*CrPpc1*, *CrPpc2*), each of which is transcribed *in vivo* and encodes a fully active, recombinant PEPC that lacks the regulatory, N-terminal seryl-phosphorylation domain typifying the vascular-plant enzyme. These distinct catalytic subunit-types differ with respect to their (a) predicted molecular mass (~108.9 (*CrPpc1*) versus ~131.2 kDa (*CrPpc2*)) and critical C-terminal tetrapeptide; and (b) immuno-reactivity with antisera against the p102 and p130 polypeptides of *S.minutum* PEPC1/PEPC2 and PEPC2, respectively. Only the *Ppc1* transcript encodes the p102 catalytic subunits common to both Class-1 and Class-2 enzyme-forms in *C.reinhardtii*. We studied the distribution of these two encoded catalytic subunits in the minor Class-1 and predominant Class-2 PEPC enzyme-forms, the latter of which is a novel high-molecular-mass, hetero-oligomeric complex containing both *CrPpc1* (p109) and *CrPpc2* (p131) polypeptides. The Class-1 enzyme, however, is a typical PEPC homotetramer comprised solely of p109. The steady-state transcript levels of both *CrPpc1/2* are coordinately up-/down-regulated by changes in [CO_2] or [NH_4^+] during growth, and generally mirror the response of cytoplasmic glutamine synthetase (*Gs1*) transcript abundance to changes in inorganic [N] at 5% CO_2. We also documented that the amount of both *CrPpc1/2* catalytic subunits is up-/down-regulated by varying levels of NH_4^+ supplied to the culture medium. To our knowledge, these collective findings provide the first molecular insight into the *Ppc* genes and corresponding PEPC catalytic subunits in any eukaryotic alga.

Keywords: *PEP carboxylase, Chlamydomonas reinhardtii, green microalgae*

INTRODUCTION

Phosphoenolpyruvate carboxylase (PEPC [Ppc]; E.C. 4.1.1.31) is a ubiquitous cytoplasmic enzyme in vascular plants, and is also widely distributed among archaeal, bacterial, cyanobacterial and unicellular green-algal species (Chollet et al., 1996; Sánchez and Cejudo, 2003; Izui et al., 2004). It catalyzes the irreversible β-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO_3^- and Me^{2+} to yield inorganic phosphate and oxaloacetate (OAA), and thus is involved intimately in C_4-dicarboxylic acid metabolism in these organisms. While the enzyme is clearly best known for its cardinal roles in C_4-photosynthesis and *Crassulacean* acid metabolism (CAM), green-plant PEPC has also been widely studied in a diverse array of non-photosynthetic contexts. During the past 10 to 15 years an impressive list of advances in vascular-plant and prokaryotic PEPC research has been generated (Chollet et al., 1996; Izui et al., 2004). In marked contrast to this wealth of information on vascular-plant and prokaryotic PEPC, until recently there was little or no biochemical or molecular insight into the green-microalgal enzyme. However, starting in 1996 a series of detailed biochemical studies of the PEPC enzyme-forms purified from two unicellular green algae, *Selenastrum minutum* and *Chlamydomonas reinhardtii*, were published (Rivoal et al., 1996, 1998, 2002). These collective findings revealed that the lesser abundant Class-1 PEPCs are homotetramers of ~102-kDa catalytic subunits (p102). However, in neither of these cases have the various component subunits of the Class-1 and Class-2 enzymes been identified in rigorous molecular terms. Given this complete lack of molecular insight into the various component subunits of the novel Class-1 and Class-2 PEPC enzyme-forms in the green microalgae, we first set out to identify the *Ppc* gene(s) encoding the catalytic polypeptides of these two enzyme-classes in *C.reinhardtii*. As a result of these initial efforts we have cloned and characterized *two novel* and distinct *Ppc* genes in this unicellular green alga. The

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respective sequences of *CrPpc1* and *CrPpc2* genes were deposited in GenBank under accession numbers of AY517644 and AY517643. We also presented biochemical insight into the effects of a varying supply of inorganic-N to *C.reinhardtii* cultures on the CrPpc1/2 and Class-1/-2 protein levels.

MATERIALS AND METHODS

Cells and growth conditions. *C.reinhardtii* cells (strains CC-125, CC-1883, and CC-1021/2137 were cultured at 25°C in continuous light (~100 μmol m^{-2} s^{-1}, 400–700 nm) in HS medium.

Cloning of the CrPpc1 and CrPpc2 genes. cDNAs of the *C.reinhardtii Ppc* genes, were isolated as described by Mamedov et al. (2005). Both *Ppc* cDNA ORFs were cloned into pBluescript II(-) phagemid vector (Stratagene) designated as *CrPpc1* (AY517644) and *CrPpc2* (AY517643).

Sequence-alignment, phylogenetic and gene-structure analyses. Deduced amino-acid-sequence alignments of *C.reinhardtii* Ppc1 and Ppc2, together with representative plant and prokaryotic PEPCs, were performed using VECTOR NTI 7.0 software. Likewise, a phylogenetic tree was constructed with predicted, full-length PEPC amino-acid sequences aligned using the ClustalX program (version 1.81) with manual adjustments. A distance matrix for the alignment was calculated, and an unrooted tree was constructed using the Protdist (with JTT model) and Neighbor programs of the PHYLIP package (version 3.62), respectively (Felsenstein, 1996). Bootstrap analysis was performed with 1000 replications and the tree was visualized using TreeView 32 software.

Construction and purification of recombinant, His_6-tagged C.reinhardtii Ppc1 (rCrQNTG) and Ppc2 (rCrRNTG) proteins. PCR, plasmid construction, bacterial transformation, recombinant PEPCs expression and purification of recombinant proteins were performed as described by Mamedov et al. (2005).

Production and affinity-purification of isoform-specific, CrPpc1 and CrPpc2 peptide antibodies. Antisera against both *Chlamydomonas* PEPC catalytic subunits (CrPpc1 [p109], CrPpc2 [p131]), hereafter designated CrPpc1/2 N-pAbs, were generated using as described elsewhere (Mamedov et al., 2005).

Native-, SDS-PAGE, in-gel PEPC assay, and immunoblotting. Native-, SDS-PAGE, in-gel PEPC assay and immunoblotting were performed as described previously (Mamedov et al., 2005). PEPC activity in the crude supernatant fractions was assayed spectrophotometrically at pH 8.4.

RNA and DNA hybridizations. Northern and Southern blot analyses were performed as described elsewhere (Mamedov et al., 2005).

RESULTS AND DISCUSSION

Cloning and sequence analyses of two novel and distinct Ppc genes in C.reinhardtii

The corresponding *CrPpc1/2* cDNAs were isolated, sequenced and cloned, and their sequences deposited in GenBank under nucleotide accession nos. AY517644/AY517643, respectively. The nucleotide sequences of these two distinct *C.reinhardtii Ppc* transcripts share 46% identity within both the open reading frame (ORF) and the 3'-UTR. A selected alignment of the deduced, full-length amino-acid sequences of these two polypeptides, along with representative plant and prokaryotic PEPCs, is depicted in Figure 1. Of special note is that both CrPpc1 and CrPpc2 (i) lack the N-terminal seryl-phosphorylation domain that typifies the green-plant enzyme; (ii) harbor all the conserved subdomains that contribute essential residues to the active site (e.g. see boxes I–III in Figure 1); and (iii) contain the conserved, hydrophobic C-terminal domain that participates in both negative allosteric regulation and maximal catalysis by PEPC. In contrast, the deduced CrPpc1 and CrPpc2 polypeptides also differ significantly in a number of important respects: they share only a 30% overall amino-acid sequence; deduced amino-acid sequence of CrPpc1 has a green-plant-like QNTG motif at its extreme C-terminus, whereas CrPpc2 has a non-archaeal, prokaryotic-like motif (RNTG) at its carboxy-terminus; near the N-terminus only the deduced sequence of CrPpc1 agrees favorably (87% identity between Gln16–Arg30 (Figure 1)) with the 15 aminoacid-residue sequence of the 102 kDa catalytic polypeptide of *C.reinhardtii*, Class-2 PEPC determined directly by N-terminal microsequencing (Rivoal et al., 1998). This notable finding indicates that only this specific *Ppc* transcript encodes the p102 catalytic subunits common to both the Class-1 and Class-2 enzyme-forms in *C.reinhardtii*. Finally, the predicted molecular mass of the 974-residue CrPpc1 (CrQNTG) polypeptide is 108 887 Da, whereas that of CrPpc2 (CrRNTG) is 131 218 Da. Notably, the deduced molecular size of CrRNTG, encompassing 1221 amino acids, is the largest PEPC catalytic subunit reported to date (Figure 1). Phylogenetic analysis of CrPpc1/2 and a number of other PEPCs revealed a clustering into three general groups (Mamedov et al., 2005): (i) the typical vascular-plant enzymes, of which CrPpc1, with its 109 kDa *Mr* and C-terminal QNTG tetrapeptide, is a distant member; (ii) the recently

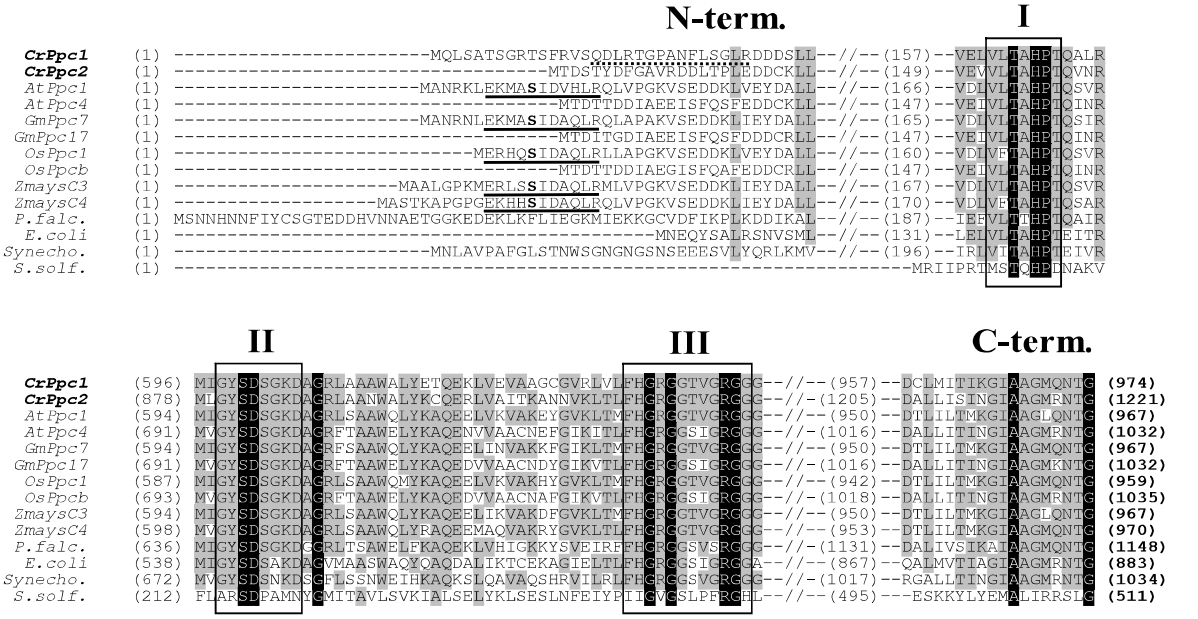

Figure 1. Selected amino-acid-sequence alignments of deduced regulatory and catalytic subdomains in PEPC from *C.reinhardtii* and representative vascular-plant and prokaryotic species. The abbreviated name of each aligned sequence is as follows: CrPpc1, *C.reinhardtii* Ppc1 (CrQNTG); CrPpc2, *C.reinhardtii* Ppc2 (CrRNTG); AtPpc1, *Arabidopsis thaliana* Ppc1; AtPpc4, *A.thaliana* Ppc4 (C-terminal RNTG ['bacterial-type']); GmPpc7, *Glycine max* Ppc7; GmPpc17, *G.max* Ppc17 (C-terminal KNTG ['bacterial-type']); OsPpc1, *Oryza sativa* Ppc1; OsPpcb, *O.sativa* Ppc-b (C-terminal RNTG ['bacterial-type']); ZmaysC3, *Zea mays* C3-form Ppc; ZmaysC4, *Z.mays* C4-form Ppc; P.falc., *Plasmodium falciparum* Ppc; E.coli, *E.coli* Ppc; Synecho., *Synechocystis sp.* PCC6803 Ppc; S.solf., *Sulfolobus solfataricus* Ppc (archaeal). Boxes I–III indicate conserved subdomains essential for catalysis by PEPC (Izui et al., 2004).

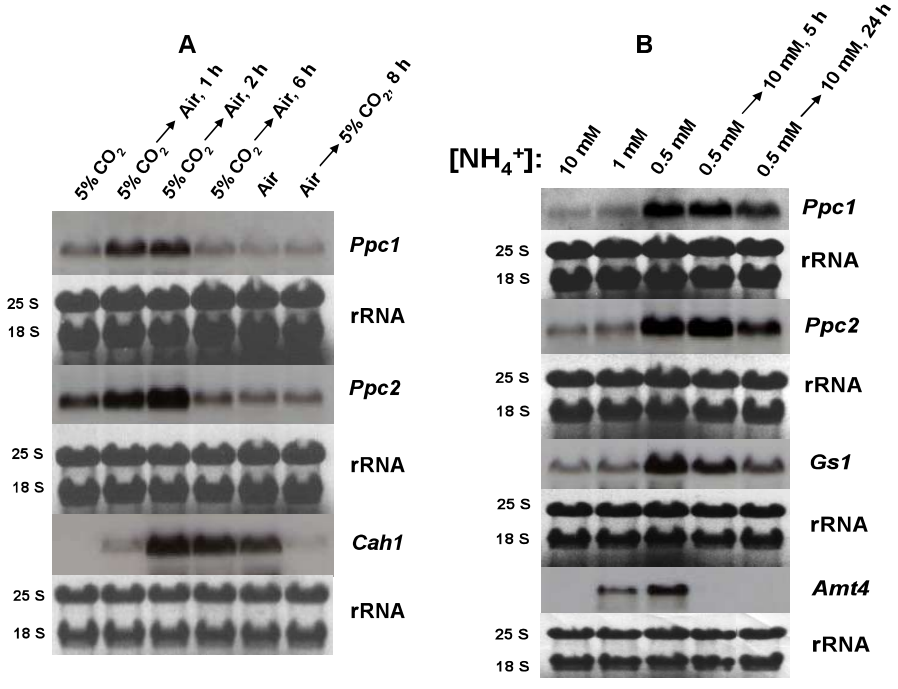

Figure 2. **A:** Northern blot analysis of *Ppc1, Ppc2*, and a Ci-responsive control transcript (*Cah1*) in *C.reinhardtii* cells grown in high/low levels of CO_2. **B:** Northern blot analysis of *Ppc1, Ppc2*, and N-responsive control transcripts (*Gs1* and *Amt4*) in *C.reinhardtii* cells grown in high/low levels of NH_4^+, regulated within 5 h of growth under these N-sufficient conditions.

described, 'bacterial-type' plant PEPCs reported, to date, in *Arabidopsis* (AtPpc4), rice (OsPpc-b) and soybean (GmPpc17), of which *C.reinhardtii* Ppc2, with its C-terminal RNTG tetrapeptide, is a distant member; and (iii) the archaeal and (cyano)bacterial PEPCs, which consistent with PEPC's multi-faceted functional diversity in green plants, similarly small multigenic Ppc families have been reported previously (Chollet et al., 1996). At the level of overall gene structure, almost all vascular plant *Ppc* genes have a highly conserved genomic structure composed of approximately 10 exons interrupted by introns, regardless of whether they are from C_3 (e.g. AtPpc1 (Mamedov et al., 2005)), C_4 or CAM plants (Chollet et al., 1996). The deduced gene structure of *C.reinhardtii Ppc2*, with its 21 exons, is thus most similar to these 'bacterial-type' plant *Ppc* genes (Mamedov et al., 2005). The longer introns found in *CrPpc2* make it the largest *Ppc* gene (12.7 kb) identified to date.

Steady-state transcript analysis of CrPpc1, CrPpc2, and a known inorganic C-responsive gene (Cah1) in C.reinhardtii cells grown in high/low levels of CO_2 and 10 mM NH_4^+

As shown in Figure 2A, when *C.reinhardtii* cells grown at 5% CO_2 were transferred to a low-CO_2 condition (ambient air), the transcript levels of both *Ppc* genes increased transiently within 1 h, reached a maximum after 2 h, and then declined following 6 total hours of acclimation to these limiting-CO_2 conditions. In addition, the levels of both *Ppc* transcripts were down-regulated in cells grown in air relative to high CO_2-grown cells. However, when the air-grown cells were transferred to high-CO_2, the transcript levels of both *Ppc* genes remained largely unchanged after 8 h of accumulation (Figure 2A). These collective results indicate that both *CrPpc1* and *CrPpc2* are CO_2-responsive genes (Chen et al., 1997; Asamizu et al., 2000; Mamedov et al., 2001), and their steady-state transcript levels are up-/down-regulated by varying levels of CO_2 supplied to the growth medium. The similar expression responses observed for *CrPpc1* and *CrPpc2* under these various perturbations in [Ci] suggest the possibility of a similar mechanism regulation at the transcriptional level. *Cah1* which encodes a periplasmic α-carbonic anhydrase (α-CA (Fukuzawa et al., 2001)) was used as a Ci-responsive reference transcript whose expression is related directly to the induction of the CCM in *C.reinhardtii* cells. As shown in Figure 2A, little or no accumulation of the *Cah1* transcript was detected under high-CO_2 conditions, in contrast to *Ppc1* and *Ppc2*. However, the level of *Cah1* mRNA increased within 1 h of low-CO_2 acclimation and attained its maximum after 2 h. Notably, during this transient period *Ppc1* and *Ppc2* responded in a similar manner. In contrast, there was a striking down-regulation of *Cah1* expression when air-grown cells were transferred to 5% CO_2 for 8 h, whereas *Ppc1* and *Ppc2* levels were essentially unchanged after this relatively brief period of acclimation to high CO_2.

Steady-state transcript analysis of CrPpc1, CrPpc2, and known inorganic N-responsive genes (Gs1 and Amt4) in C.reinhardtii cells grown in high/low levels of NH_4^+ at 5% CO_2

PEPC is a major anaplerotic enzyme in the green microalgae, especially in the context of replenishing citric-acid-cycle intermediates, such as 2-oxogluta- rate, consumed during the assimilation of ammonia by the GS/GOGAT cycle (Huppe and Turpin, 1994). It was thus of considerable relevance to assess the effects of varying levels of inorganic N, initially supplied as NH_4Cl, on the expression of the *Ppc1* and *Ppc2* genes in *C.reinhardtii*, and any concomitant changes in total cellular PEPC activity. As a complement to these *Ppc* northern analyses, the expression of two distinct, inorganic-N responsive reference transcripts was also monitored, namely *Amt4* and *Gs1*. Notably, the steady-state transcript levels of *Amt4* are increased specifically and dramatically under N-limiting conditions. *Gs1* represents the sole gene in *C.reinhardtii* that encodes the cytoplasmic isoform of GS, an obvious component of the N-assimilating GS/GOGAT cycle. The transcript levels of *Gs1* are significantly down-regulated under conditions of excess NH_4^+ (Chen and Silflow, 1996). As shown in Figure 2B, the transcripts for *Ppc1*, *Ppc2*, and *Gs1* were all readily detected in cells grown in 10 mM NH_4^+ and air enriched with 5% CO_2 (also see the related *Ppc* data in Figure 2A). In contrast, the exquisitely N-sensitive *Amt4* gene was not expressed under these conditions of N-sufficiency. With growth in decreasing levels of NH_4^+, all four genes were progressively up-regulated, with the maximal increase in steady-state transcript abundance occurring at 0.5 mM. Clearly, *Ppc1* and *Ppc2* are N-responsive genes in *C.reinhardtii*, and their expression profiles in response to decreasing NH_4^+ levels from 10 to 0.5 mM at 5% CO_2 mirrored that of *Gs1*. When the low-N grown cells were re-supplied directly with 10 mM NH_4^+, there was a modest but detectable down-regulation of *Ppc1*, *Ppc2* and *Gs1* expression within 24 h (Figure 2B). In contrast, the exquisitely N-sensitive *Amt4* gene was once again specifically and completely down-regulated. It is noteworthy that while concomitant changes in total *in vitro* PEPC specific activity (on a soluble protein basis) general-

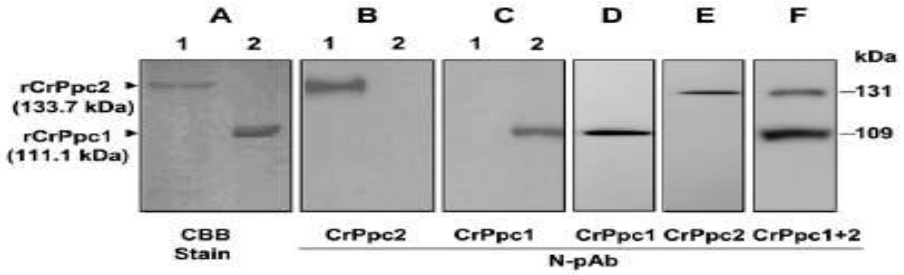

Figure 3. Immunospecificity of affinity-purified, N-terminal CrPpc1/2 peptide antibodies against the recombinant CrPpc1/2 fusion proteins and *C.reinhardtii* crude cell extracts following SDS–PAGE. (A) Coomassie Blue Staining of His6-tagged recombinant CrPpc2 (lane 1) and CrPpc1 (lane 2) marker proteins; (B, C) Immunoblot analysis of recombinant CrPpc2 (lanes 1) and CrPpc1 (lanes 2) proteins with CrPpc2 and CrPpc1 N-pAbs, respectively; (D-F) Immunoblots of *C.reinhardtii* (TAP-grown) crude soluble extracts with CrPpc1/2, and 1 + 2 N-pAbs, respectively.

ly paralleled those for *Ppc1* and *Ppc2* transcript abundance with decreasing [NH_4^+] from 10 to 0.5 mM.

Heterologous expression, and activity- and immunoblot-analysis of the C.reinhardtii recombinant CrQNTG (Ppc1) and CrRNTG (Ppc2) proteins

The expressed recombinant proteins were found to be highly soluble and effectively purified from clarified cell extracts by Ni^{2+}-immobilized metal affinity-chromatography (IMAC) as evidenced by SDS-PAGE analysis (Figure 3A). As depicted in Figures 3A and B, the *Chlamydomonas* recombinant Ppc1 and Ppc2 polypeptides were produced as 994-residue/~111 kDa and 1244-residue/~134 kDa fusion proteins, respectively, harbored a His_6-tag in their N-terminal extension in order to effect their separation from the resident *E.coli* PEPC. The specific activities of recombinant CrQNTG and CrRNTG were ~25 and ~22 mol $min^{-1}mg^{-1}$protein, respectively. These values are similar to the 18 to 22 U mg^{-1} specific activities reported for the Class-1 and Class-2 PEPCs purified from *C.reinhardtii* cell extracts (Rivoal et al., 1998). In marked contrast, affinity-purified polyclonal antibodies raised against the p102 catalytic subunit common to both green-algal PEPC1 and PEPC2, and the interacting p130 polypeptide of PEPC2 from *S.minutum* (Rivoal et al., 1996, 2002) reacted immuno-specifically with rCrQNTG or rCrRNTG, respectively (data not shown). The finding that the fully active ~134-kDa recombinant CrRNTG protein reacts immuno-specifically with anti-*S.minutum* p130 antibodies indivate that p130 is a *novel*, active PEP-carboxylase polypeptide in the green microalgae. As a result of these collective data, we conclude that there are likely two distinct PEPC catalytic subunit-types in the unusual but dominant Class-2 enzyme-complexes in the unicellular green algae, contributed by both p102 (e.g., CrPpc2) and the interacting p130 polypeptide (e.g., CrPpc1). The affinity-purified CrPpc1/2 N-pAbs were found to immunoreact specifically with the purified, recombinant parent protein (Figure 3, panel B-F) for which they were raised. Likewise, soluble extracts with these affinity-purified N-pAbs resulted in the detection of a single p109 or p131 polypeptide which essentially co-migrated with the immunosignal from the corresponding react specifically with their divergent polypeptide targets upon denaturing immunoblot analysis, both in purified recombinant form and in soluble protein extracts from *C.reinhardtii* cells.

Denaturing immunoblot analysis of extracts from C.reinhardtii cells grown in various concentrations of NH_4^+ at 5% CO_2 or in TAP medium

As we discussed above the *CrPpc1/2* transcript levels to be coordinately up-/down-regulated under varying levels of NH_4^+ supplied to a photoautotrophic culture medium. As shown in Figure 4A, the corresponding CrPpc1/2 polypeptide levels are also up-regulated as the initial supply of NH_4Cl decreased from 10 to 0.5 mM. However, within 5 h after re-supply of 10 mM NH_4Cl to the N-deficient cells, the CrPpc1/2 levels reverted back nearly to those observed in high-N grown cells. This is in striking contrast with the corresponding transcript levels that are up-regulated in low-N cells, and remain largely elevated even after 24 h of ammonium re-supply. *In vitro* total cellular PEPC activity measurements showed an increase from ~0.035 to ~0.090 U mg^{-1} protein when the supplied [NH_4^+] was decreased from 10 to 0.5 mM, which returned to ~0.035 U mg^{-1} within 5 h of re-supplying 10 mM NH_4Cl (data not shown).

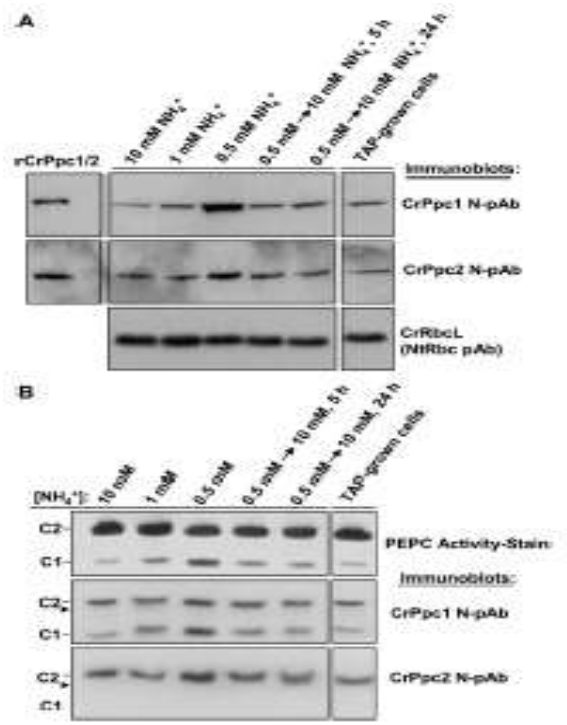

Figure 4. Immunoblot analysis and in-gel PEPC activity-staining of soluble extracts from *C.reinhardtii* cells grown at varying levels of NH_4Cl in air enriched to 5% CO_2 or in TAP medium in air. (A) Soluble proteins (22 µg) from *C.reinhardtii* cell extracts were separated by SDS-PAGE and immunoblotted with CrPpc1/2 N-pAbs or tobacco Rubisco pAb (used to detect the large subunit of *Chlamydomonas* Rubisco (CrRbcL)). (B) 1.25 mU of total PEPC activity from soluble cell extracts was separated by native-PAGE and either stained for in-gel PEPC activity in the presence of PEP as in Figure 1B, or immunodecorated with CrPpc1/2 N-pAbs.

C.reinhardtii cultures grown mixotrophically in acetate-containing TAP medium with 7.5 mM NH_4Cl exhibited CrPpc1/2 polypeptide levels (Figure 4A) and total cellular PEPC activity (~0.029 U mg^{-1}) approximating those in 5% CO_2-aerated cells grown at 10 mM NH_4^+ in HS medium. These complementary results show that the levels of the divergent CrPpc1/2 polypeptides generally mirror the corresponding changes in total cellular PEPC activity under the high-/low-N growth conditions examined.

1D native-PAGE and SDS-PAGE, combined with immunoblotting, establish the novel presence of the divergent CrPpc1/2 catalytic subunits in the hetero-oligomeric Class-2 PEPC complex

Non-denaturing electrophoretic analysis of *C.reinhardtii* soluble cell extracts, exploiting the Rubisco holoenzyme as an endogenous, ~550-kDa marker, revealed that both the single CrPpc2 N-pAb immunosignal and uppermost CrPpc1 N-pAb signal co-migrated with the predominant, Class-2 PEPC activity-band, irrespective of the growth conditions examined (Figure 4B). In contrast, the lower CrPpc1 N-pAb immunosignal co-migrated with the Class-1 PEPC activity-band. These provocative results, together suggest that the divergent CrPpc1/2 catalytic subunits co-exist in the *C.reinhardtii* Class-2 PEPC complex. Interestingly, the relative proportion of Class-1 to Class-2 PEPC in *C.reinhardtii* was highest in 0.5 mM NH_4Cl-grown cells (Figure 4B). Thus, the obtained data collectively document the first definitive example of a eukaryotic PEPC holoenzyme-complex containing two, structurally distinct catalytic subunit-types, p109 and p131. Equally surprising is the realization that the former, CrPpc1 polypeptide is most closely related phylogenetically to the ~110-kDa PEPC-subunit of vascular plants, whereas the latter, CrPpc2 catalytic subunit is most homologous to the enigmatic "bacterial-type" plant PEPCs.

CONCLUDING REMARKS

To our knowledge, the above collective findings provide the first molecular insight into the two novel and distinct *Ppc* genes, and corresponding PEPC catalytic subunits, in *C.reinhardtii* or any other eukaryotic algae. It is now evident that *CrPpc1*, which encodes a fully functional, ~109-kDa PEPC polypeptide lacking the vascular-plant N-terminal phosphorylation domain, is the specific gene whose protein-product (CrQNTG) corresponds to the p102 catalytic subunit common to both the homotetrameric Class-1 and heteromeric Class-2 PEPC-forms in *C.reinhardtii*. Similarly, the divergent but highly active, ~131-kDa CrRNTG catalytic subunit, encoded by the unusual, ~12.7-kb *CrPpc2* gene, is also void of the "typical" N-terminal phosphorylation domain, and likely is equivalent to the interacting p130 polypeptide that is unique to the green-algal Class-2 PEPC complex. Thus our biochemical findings implicate the novel presence of two distinct PEPC catalytic subunit-types in the unusual but dominant Class-2 enzyme-complexes in the unicellular green algae. Class-2 PEPC enzyme-forms is a novel high-molecular-mass, hetero-oligomeric complex which containing both CrPpc1 (p109) and CrPpc2 (p131) polypeptides. The Class-1 enzyme, however, is a typical PEPC homotetramer comprised solely of p109.

At the level of expression, both of these "anaplerotic" *Ppc* genes in *C.reinhardtii* are coordinately responsive to changes in inorganic-C and -N levels during growth and they generally mirror the response of *Gs1* transcript abundance to changes in $[NH^{4+}]$ at high CO_2. This correlation between *Ppc1*/*Ppc2* and *Gs1* expression in *Chlamydomonas*

provides the first direct molecular evidence in support of previous physiological and biochemical studies that highlighted a key anaplerotic, non-photosynthetic role for PEPC in overall N-assimilation by the GS/GOGAT cycle in green microalgae.

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