A Nuclear-Encoded Form II RuBisCO in Dinoflagellates

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The chloroplasts of most dinoflagellates are unusual in that they are surrounded by three membranes and contain the carotenoid peridinin. The ribulose-1,5-bisphosphate carboxylase–oxygenase (RuBisCO) in dinoflagellate chloroplasts was found here to also be unusual. Unlike other eukaryotes, dinoflagellates containing peridinin use a form of RuBisCO (form II) previously found only in some species of proteobacteria. Furthermore, this RuBisCO is not encoded in the chloroplast DNA, as is the case in other organisms, but is encoded by the nuclear DNA. The unusual nature of this enzyme and location of its gene support the idea that dinoflagellate chloroplasts may have had a distinctive evolutionary origin.

The most usual form of the enzyme RuBisCO (termed form I) is a protein comprising eight large and eight small subunits. It is common in both prokaryotes and eukaryotes, and differences in the amino acid sequences of up to 40% have been found between the enzymes of organisms lacking chlorophyll b (such as rhodophytes) and organisms containing chlorophyll b (such as cyanobacteria and chlorophytes) (*1*). A second and distinctly different form of RuBisCO also exists (form II), and it is composed of only large subunits that share limited (25 to 30%) sequence identity with the form I large subunit (*2*). This form of the enzyme has not previously been found in chloroplasts.

Two-dimensional gels of proteins extracted from the marine dinoflagellate *Gonyaulax polyedra* revealed a group of four protein isoforms that, on the basis of their size (55 kD) and high relative abundance, were considered to be good candidates for the large subunit of RuBisCO (Fig. 1A). Indeed, microsequencing of several internal peptides (75 amino acids) derived from one of the gel-purified isoforms (arrow in Fig. 1A) revealed an 80% sequence identity to form II RuBisCO of the proteobacteria *Rhodospirillum rubrum* (Fig. 1B). Protein immunoblot analysis with an antibody raised against *R. rubrum* RuBisCO (anti–form II RuBisCO) (Fig. 1C) showed immunoreactivity with the isoform sequenced and with the three other 55-kD isoforms, supporting the identification of form II based on sequence comparisons (Fig. 1B). The anti–form II RuBisCO also stained *Gonyaulax* chloroplasts in cell sections, indicating that the presence of the protein is not the result of contamination in our unialgal but not axenic cultures (*3*). No reactivity was observed with an antibody raised against the form I large subunit of the higher plant *Nicotiana*, suggesting that only the form II enzyme is present in *Gonyaulax*.

To confirm the identity of the *Gonyaulax* RuBisCO as a form II enzyme, we amplified a DNA fragment that corresponds to the sequenced *Gonyaulax* RuBisCO by polymerase chain reaction (PCR) (*4*). Oligonucleotide primers were designed on the basis of amino acid sequences present in both the *Gonyaulax* microsequence and in the *Rhodospirillum* RuBisCO (*5*), and these primers were used to amplify PCR products of similar size from both a *Rhodospirillum* RuBisCO DNA clone and a *Gonyaulax* complementary DNA (cDNA) library (Fig. 2A). The nucleotide sequences of the PCR fragments were not identical, however, as the *Rhodospirillum* RuBisCO PCR product hybridized poorly to a *Gonyaulax* PCR probe at high stringency (Fig. 2B). We sequenced several RuBisCO clones obtained by screening the *Gonyaulax* cDNA library with the homologous PCR product. The deduced amino acid sequence of a partial length RuBisCO cDNA shared 62% sequence identity with the *Rhodospirillum* enzyme over 485 amino acids (Fig. 2D) and only 23% identity with *Nicotiana* RuBisCO.

RuBisCO clones were found in the cDNA library at high frequency (0.22%), suggesting that this gene is encoded by the nucleus. This is supported by an analysis of the *Gonyaulax* cDNA sequence; like other cDNAs isolated from the library (*6*), the clone is glysine-cysteine (GC) rich (60%) and is followed by a 190–base pair (bp) 3′ noncoding region and a 12- to 20-residue polyadenylate [poly(A)] tail. We confirmed the nuclear location of the RuBisCO gene in two ways: by PCR, where amplified products could be detected with genomic DNA but not with chloroplast DNA as a template (Fig. 2B), and by slot blot analysis, which revealed that the hybridization pattern of the RuBisCO gene probe to genomic and chloroplast DNA is different from that of a heterologous chloroplast *atpB* gene probe to the same samples (Fig. 2C). Although a naturally occurring, nuclear-encoded large RuBisCO subunit gene has not been previously observed, there are numerous examples of nuclear-encoded RuBisCO small subunit genes (*7*), and the large subunit gene can function normally when relocated to the nucleus if a suitable transit peptide is provided for the protein (*8*).

There are two possible explanations for the presence of the form II RuBisCO in

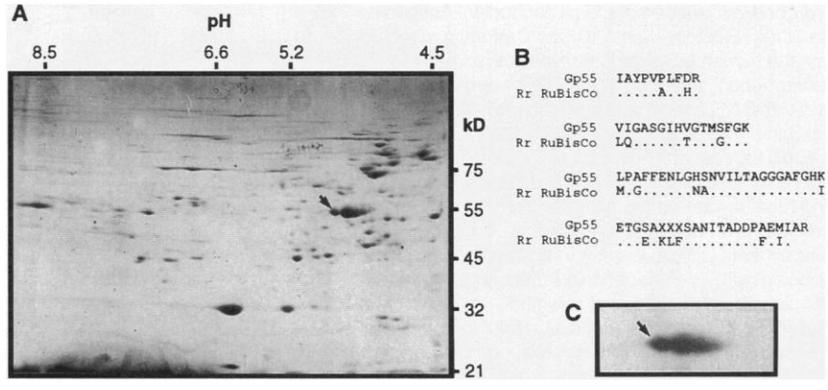

Fig. 1. *Gonyaulax* contains only the form II RuBisCO. (**A**) Two-dimensional gel electrophoresis analyses of total *Gonyaulax* protein stained with Coomassie blue shows the presence of several abundant protein isoforms with a size (55 kD) appropriate for the large RuBisCO subunit (*19*). (**B**) Microsequencing (*20*) of one of the isoforms [arrow in (A)] revealed a strong homology to the *R. rubrum* (Rr) RuBisCO. Abbreviations for the amino acid residues are given in (*18*). X indicates an unsequenced residue, and dots signify identity. (**C**) Protein immunoblot analysis (*21*) shows that antibody raised against *R. rubrum* RuBisCO cross-reacts with all 55-kD isoforms in the *Gonyaulax* extracts.

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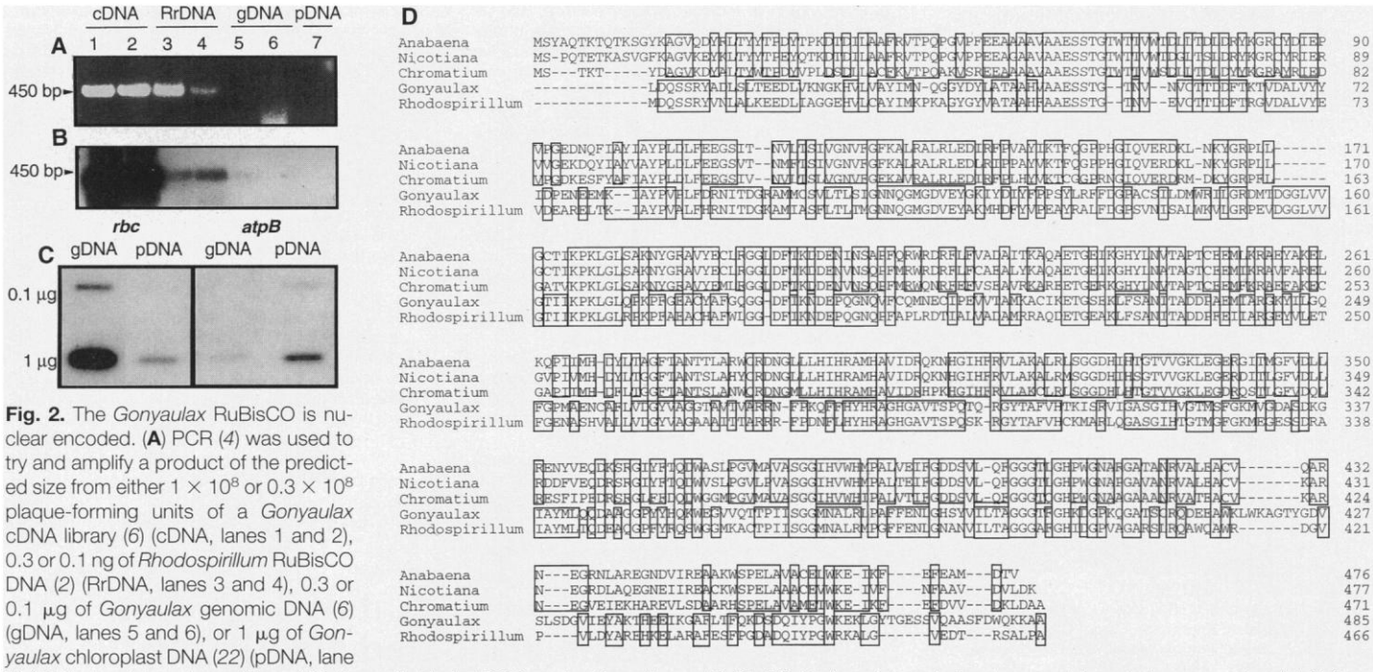

Fig. 2. The *Gonyaulax* RuBisCO is nuclear encoded. (**A**) PCR (*4*) was used to try and amplify a product of the predicted size from either 1×10^8 or 0.3×10^8 plaque-forming units of a *Gonyaulax* cDNA library (*6*) (cDNA, lanes 1 and 2), 0.3 or 0.1 ng of *Rhodospirillum* RuBisCO DNA (*2*) (RrDNA, lanes 3 and 4), 0.3 or 0.1 µg of *Gonyaulax* genomic DNA (*6*) (gDNA, lanes 5 and 6), or 1 µg of *Gonyaulax* chloroplast DNA (*22*) (pDNA, lane 7). (**B**) Under stringent conditions (*23*), the radiolabeled PCR product from the *Gonyaulax* library hybridized weakly to the PCR fragment amplified from either the *Rhodospirillum* RuBisCO (lanes 3 and 4) or genomic DNA (lanes 5 and 6). No signal was seen when chloroplast DNA was used as the template (lane 7). (**C**) A slot blot (*24*) of 0.1 and 1 µg of genomic (*6*) and chloroplast DNA (*22*) shows that these DNA preparations hybridized differently to a *Gonyaulax* RuBisCO (*rbc*) and to a heterologous chloroplast gene probe (*atpB* from *Tetraselmis maculata*). (**D**) A RuBisCO clone was sequenced on both strands with the dideoxy chain termination method (kit from Pharmacia), and the deduced amino acid sequence was aligned with the sequence of *Rhodospirillum* and *Nicotiana* RuBisCOs (*18, 25*).

Gonyaulax, both of which have evolutionary implications. One possibility is that dinoflagellate chloroplasts with the form II RuBisCO had an evolutionary origin independent of higher plant chloroplasts. This has been suggested on the basis of pigment composition (*9*) because although most dinoflagellates contain the carotenoid peridinin characteristic of this algal division (*10*), some species contain pigments similar to those found in other algae (*11, 12*).

To address this first explanation experimentally, we tested *Gyrodinium aureolum*, a dinoflagellate species that lacks peridinin (*12*), for the form II RuBisCO. We reasoned that the presence of a distinctive pigment together with a rare RuBisCO would suggest that both were present in an ancestral symbiont. Protein immunoblot analysis showed that extracts from three different peridinin-containing dinoflagellates stained with the anti–form II RuBisCO, whereas a species lacking peridinin did not (Fig. 3). If RuBisCO is an indicator of the plastid's origin (*13*), then the ancestral dinoflagellate chloroplast must have been an ancestor of proteobacteria and cyanobacteria (*14*), because modern day proteobacteria do not contain chlorophyll a and do not evolve O_2. It is unlikely that the dinoflagellate chloroplast is derived directly from a eukaryotic symbiont, because only prokaryotes have been observed to contain the form II RuBisCO.

The other explanation for the presence of the form II RuBisCO gene is that the progenitor to modern algae had three different genes (two different form I and one form II sequence) and lost all but one. Peridinin would then have evolved subsequently in the dinoflagellate lineage. There is strong evidence that chloroplasts were derived from cyanobacteria (*15*), whereas mitochondria were derived from photosynthetic proteobacteria (*16*). Thus, the ancestral eukaryote could have contained not only a cyanobacterial-type form I RuBisCO but also proteobacterial form I and form II RuBisCOs (*17*). All three branches of current RuBisCO phylogeny (dinoflagellates, rhodophytes, and higher plants) could thus be derived from this ancestral eukaryote by transfer of the RuBisCO gene between subcellular compartments. In this scheme, algae containing chlorophyll b and higher plant chloroplasts have retained their original form I sequence, whereas the mitochondria have lost both the form I and form II sequences. In the plastids lacking chlorophyll b, the original cyanobacterial RuBisCO would have been replaced with the mitochondrial form I sequence and the form II RuBisCO sequence lost. The dinoflagellates would have transferred the mitochondrial form II sequence to the nucleus and lost both the mitochondrial and plastid form I sequences. We note that this hypothesis would explain why RuBisCO molecular phylogenies do not always agree with those of other sequences, such as chloroplast ribosomal RNA (rRNA) (*1*).

In conclusion, we have shown that dinoflagellate RuBisCO is unlike that of other eukaryotes and that the gene coding for the protein is found in the nucleus rather than in the chloroplast. This unusual situa-

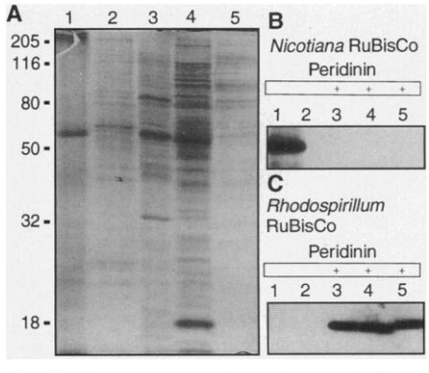

Fig. 3. The presence of the form II RuBisCO correlates with the presence of peridinin. (**A**) SDS-PAGE of 50-µg protein samples (*26*) isolated from the higher plant *Nicotiana tobaccum* (lane 1), as well as from the dinoflagellates *Gyrodinium aureolum* (lane 2), *G. polyedra* (lane 3), *Amphidinium pacificum* (lane 4), and *Pyrocystis lunula* (lane 5). The gel was stained with Coomassie brilliant blue. Molecular sizes are indicated to the left in kilodaltons. (**B**) Reaction of the same samples with an anti–*Nicotiana* RuBisCO (*21*). (**C**) Reaction of the same samples with an anti–*Rhodospirillum* RuBisCO.

tion represents strong evidence for a distinctive evolutionary origin of dinoflagellate chloroplasts, although a comparison of their plastid rRNA sequences with those of extant organisms will be required to fully resolve the issues raised here. However, it is clear that the dinoflagellate RuBisCO represents a form of the enzyme not previously found in chloroplasts.

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4. A Perkin-Elmer model 9600 PCR was run for 35 cycles of 1 min each at 95°C, 45°C, and 72°C [M. Innis, D. Gelfand, J. Sninsky, T. White, *PCR Protocols* (Academic Press, San Diego, CA, 1990)].
5. The oligonucleotides synthesized corresponded to the peptides ANITA (5′-GCNAAYATHACNGCNGAYGA-3′) and GGGAF (5′-TGNCCRAANGCNCCNCCNCC-3′) (*18*).
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17. The extant proteobacteria *Rhodopseudomonas sphaeroides* has both form I and form II RuBisCO.
18. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
19. A protein extract from *Gonyaulax* cells (~1 mg of protein) was electrophoresed on two-dimensional gels [P. Milos, D. Morse, J. W. Hastings, *Naturwissenschaften* **77**, 87 (1990)].
20. The nitrocellulose membrane was stained with Ponceau red, and one 55-kD isoform was excised for microsequencing. Trypsin proteolytic fragments were sequenced by the Harvard Microchemistry Facility.
21. The gels were transferred to nitrocellulose [A. H. Towbin, T. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979)] and stained first with an antibody to *Rhodospirillum* RuBisCO and then with ^{125}I-labeled protein A before exposure to x-ray film. No reaction was observed with an antibody to *Nicotiana* RuBisCO used as a control.
22. DNA extracted from chloroplasts purified on Percoll gradients was purified on CsCl gradients as described [M. Schuler and R. Zielinski, *Methods in Plant Molecular Biology* (Academic Press, San Diego, CA, 1989)]. Chloroplasts were the only bodies present in the chloroplast fractions that were stained by 4′,6′-diamidino-2-phenylindole (a fluorescent DNA stain).
23. After transfer to Genescreen membranes, the samples were hybridized for 4 hours, according to the phosphate-SDS protocol of G. M. Church and W. Gilbert [*Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984)], and probed with a labeled PCR fragment (oligolabeling kit from Pharmacia).
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25. The protein sequences (accession numbers: *Anabaena*, P00879; *Chromatium*, P22849; *Rhodospirillum*, P00418; *Nicotiana*, P00876; and *Gonyaulax*, L41063) were aligned with the Geneworks software package (Intelligenetics, Mountain View, CA).
26. About 100 ml of each cell culture (~1 mg of protein) was centrifuged, and then the protein was extracted from the cell pellet by sonication and heating for 10 min in two volumes of sample buffer [U. K. Laemmli, *Nature* **227**, 680 (1970)]. Equal amounts of the extracts were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was either stained with Coomassie blue or transfered to nitrocellulose. Dinoflagellates were obtained from the Bigelow laboratories, Booth Bay Harbor, MN (*G. polyedra*); Ward Scientific, St. Catharines, Ontario (*A. pacificum*); and the North East Pacific Culture Collection, Vancouver, BC (*P. lunula* and *G. aureolum*). *Nicotiana tobaccum* samples were obtained from greenhouse-grown plants at the University of Montreal.

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Presynaptic Component of Long-Term Potentiation Visualized at Individual Hippocampal Synapses

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Long-term potentiation has previously been studied with electrophysiological techniques that do not readily separate presynaptic and postsynaptic contributions. Changes in exocytotic-endocytotic cycling have now been monitored at synapses between cultured rat hippocampal neurons by measuring the differential uptake of antibodies that recognize the intraluminal domain of the synaptic vesicle protein synaptotagmin. Vesicular cycling increased markedly during glutamate-induced long-term potentiation. The degree of potentiation was heterogeneous, appearing greater at synapses at which the initial extent of vesicular turnover was low. Thus, changes in presynaptic activity were visualized directly and the spatial distribution of potentiation could be determined at the level of single synaptic boutons.

The distinction between presynaptic and postsynaptic aspects of neurotransmission is fundamental to understanding how synaptic function is modulated, yet it is often difficult to assess (*1*). Vesicular release of neurotransmitter from presynaptic terminals is generally measured by electrophysiological recordings of postsynaptic signals (*2*). However, the variable responsiveness of the postsynaptic membrane renders it an unreliable indicator of presynaptic activity (*1*). This unreliability has given rise to ambiguity and controversy, particularly in the area of long-term potentiation (LTP) (*3*), for which the existence of a presynaptic component of enhancement is still intensely debated (*4*–*6*). Thus, alternative approaches are required to monitor changes in presynaptic activity directly. We now describe experiments in which vesicular fusion and recycling (*7*) were detected by the uptake of specific antibodies that recognize a luminal epitope of the vesicular protein synaptotagmin (*8*, *9*). Levels of activity during two sequential experimental periods were monitored separately with the use of two different antibody preparations, generated in different animal species, and were assessed by indirect immunofluorescence. This approach provided a ratiometric measure of changes

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