Comparisons of Nuclear-Encoded Small-Subunit Ribosomal RNAs Reveal the Evolutionary Position of the Glaucocystophyta

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The Glaucocystophyta (e.g., *Cyanophora paradoxa*) form a morphologically distinct group of photosynthetic protists that is primarily distinguished by its cyanelles (= plastids). To elucidate their evolutionary relationships, we determined nuclear-encoded small-subunit ribosomal RNA (SSU rRNA) coding regions for four taxa classified in the Glaucocystophyta (*C. paradoxa, Glaucocystis nostochinearum, Glaucosphaera vacuolata, Gloeochaete wittrockiana*; sensu Kies and Kremer), and these sequences were positioned within the eukaryotic phylogeny. Maximum likelihood, maximum-parsimony, and neighbor-joining phylogenetic analyses show that the Glaucocystophyta is a relatively late-diverging monophyletic assemblage within the “crown” group radiation that forms a sister group to cryptophyte algae. *Glaucosphaera vacuolata* is a red alga and lacks some cyanelle (e.g., bounding peptidoglycan wall) and host cell (e.g., cruciate flagellar roots) characters typical of glaucocystophytes. Our data are consistent with a monophyletic origin of the cyanelle in the glaucocystophytes. The distribution of photosynthetic taxa within the glaucocystophytes/cryptophytes and other lineages such as the filose amoebae/chlorarachniophytes and heterokont protists provide clues to the origin of plastids with four bounding membranes. We speculate that multiple, likely independent, secondary endosymbioses gave rise to these plastids.

Introduction

The Glaucocystophyta forms a small (approximately 13 species), isolated group of protists that includes flagellate, palmelloid, and coccoid cells whose unique set of characters has led to their classification into a distinct division or phylum (Kies 1979; Kies and Kremer 1986, 1990). Glaucocystophyta and other cyanelle-containing taxa (e.g., *Paulinella chromatophora*, Filosea; Kies 1974; Bhattacharya et al., in press) are closely associated with the theory of endosymbiosis due to the pigment composition (only chlorophyll-α, phycobilins), ultrastructure (phycobilisomes, carboxysomes, and concentric thylakoids), and peptidoglycan wall surrounding their cyanelles (except for *Glaucosphaera vacuolata*; Kies and Kremer 1990; Kraus et al. 1990). *Paulinella chromatophora* was the first protist to be identified as containing a “cyanobacterial-like endosymbiont” as its photosynthetic organelle (Lauterborn 1895). Cyanelle characters led previous authors to suggest that these organelles are good examples of a relatively undeveloped association between a nonphotosynthetic eukaryote and a modified coccoid cyanobacterium (Geitler 1959; Hall and Claus 1963). The cyanelles of some glaucocystophytes (i.e., *C. paradoxa, G. nostochinearum*) have even been raised to the rank of cyanobacterial taxa to reflect the distinctive ultrastructure and presumed polyphyletic origin of these organelles (Hall and Claus 1963, 1967). More recent molecular biological data of genome size, gene content, and gene order and phylogeny show that the *Cyanophora paradoxa* cyanelle shares a closer evolutionary relationship with plastids than with cyanobacteria (Bohnert et al. 1982; Giovannoni et al. 1988; Kraus et al. 1990; Douglas and Turner 1991; Palenik and Haselkorn 1992).

In order to investigate the evolutionary relatedness of the Glaucocystophyta “host” cell, we have determined the nucleotide sequence of the nuclear-encoded small-subunit ribosomal RNA (SSU rRNA) coding region from four taxa previously classified as glaucocystophytes (sensu Kies and Kremer 1986): *Cyanophora paradoxa* Korsh. (Kies strain), *Glaucocystis nostochinearum* Itzigs., *Glaucosphaera vacuolata* Korsh., and *Gloeochaete wittrockiana* Lagerheim. We compared these sequences to homologous coding regions from eukaryotes representing members of the plant, animal, fungal, and protist lineages. Small-subunit rRNA sequence analysis has proven to be a powerful tool for resolving phylogenetic relationships due to the relatively large size of this coding region (approximately 1,800 nucleotides [nt]), its universally conserved function in all cells, no documented

Key words: cyanelle, *Cyanophora paradoxa*, Glaucocystophyta, molecular evolution, phylogeny, small-subunit ribosomal RNA.

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case of lateral transfer, and a large existing data base for sequence comparisons (Woese 1987; Wainright et al. 1993).

Material and Methods

Total nucleic acids were isolated from unialgal cultures of *Cyanophora paradoxa*, *Glaucocystis nostochinearum*, *Glaucosphaera vacuolata*, and *Gloeochaete wittrockiana* using a CTAB protocol and cells that had been disrupted by vortexing in the presence of glass beads (Hillis et al. 1990; Surek et al. 1994). Ribosomal RNA coding regions were amplified using the polymerase chain reaction (PCR) protocols (Saiki et al. 1988) and primers complementary to the 5' and 3' termini of nuclear-encoded SSU rRNAs (Medlin et al. 1988). Single-stranded template DNA for sequencing was prepared using biotinylated amplification primers and the Dynabeads M-280 system (Dynal AS; Hultman et al. 1991). PCR products were sequenced directly (solid phase sequencing) using the dyeoxy sequencing method (Sanger et al. 1977) and a collection of oligonucleotide primers complementary to conserved regions of eukaryotic rRNAs (Elwood et al. 1985). Complete double-strand rRNA sequences were determined for *C. paradoxa* (1,807 nt), *G. nostochinearum* (1,819 nt), and *G. wittrockiana* (1,804 nt); a partial double-strand sequence was determined for *G. vacuolata* (1,723 nt). The nucleotide sequences of *C. paradoxa* (X68483), *G. nostochinearum* (X70803), *G. vacuolata* (X81902), *G. wittrockiana* (X81901), and all other rRNA sequences analyzed in this study are available from the EMBL/GenBank data bases.

The glaucocystophyte ribosomal RNA coding regions were aligned manually (Olsen 1990) with rRNA sequences from 43 eukaryotes representing animal, plant, fungal, and protistan lineages (alignment available from the authors). Sequence positions which could be simultaneously aligned in all studied taxa (1,621 nt) were used as input for the phylogenetic analyses. The maximum-likelihood method was implemented with the fastDNAm1 program (version 1.0; Olsen et al. 1994). The global search option was used with rearrangements of partial trees initially crossing one branch and rearrangements of the full tree crossing 44 branches. Additional analyses were done with the fastDNAm1 program in which 3 branch crossings were allowed during rearrangements of partial trees and 44 branch crossings were allowed during rearrangements of the full tree using three different random number seeds to start the jumbled taxon addition. From these searches, the tree with the highest log-likelihood was kept for the user-defined tree analyses (see below). A transition:transversion ratio of 2 was used (actual transition:transversion ratio = 1.7 within this data set) for all the maximum-likelihood analyses. Distance analysis of the same data set was done using a Kimura (1980) matrix as input for a neighbor-joining phylogenetic reconstruction (Saitou and Nei 1987) with jumbled taxon addition and the transition:transversion ratio of 2 (PHYLIP version 3.5; Felsenstein 1993). Maximum-parsimony analysis was done using a weighting scheme (rescaled consistency index over an interval of 1-1,000) for each site within the aligned data set (the maximum-parsimony method is improved when sites that have relatively higher rates of change are given less weight in the phylogenetic analysis; Hillis et al. 1994). Random additions of the weighted data were analyzed with a heuristic method with a branch-swapping algorithm (tree bisection-reconnection [TBR], PAUP version 3.1.1; Swofford 1993). Bootstrap analyses (100 replications) were done with the distance and maximum-parsimony methods (Felsenstein 1985).

The monophyly of the glaucocystophytes within the maximum-likelihood phylogeny was tested with user-defined tree analyses. In this method, topologies were created with the RETREE option (PHYLIP 3.5) that address three alternate hypotheses regarding the evolutionary position of the glaucocystophytes: as an independent lineage within the “crown” (Knoll 1992) group radiation, on the branch uniting them with the rhodophytes and on the branch uniting them with the chlorophytes. The log-likelihoods of these alternate trees and that of the “best” tree (i.e., found with the global search) were compared with the likelihood ratio test (LRT) of Kishino and Hasegawa (1989). The LRT uses the mean and variance of log-likelihood differences between trees taken across sites, and when this mean is >1.96 standard deviations different between any two trees, these are declared significantly different (Felsenstein 1993). This test assumes no autocorrelation between rates of divergence at different sites.

Results and Discussion

Results of maximum-likelihood, neighbor-joining and maximum-parsimony analyses of nuclear-encoded small-subunit rRNA coding regions from 47 eukaryotic taxa are summarized in figure 1. Bootstrap percentage values from neighbor-joining and maximum-parsimony analyses are shown at branches defining topological elements shared by these and the maximum-likelihood phylogeny which appear in greater than 60% of the bootstrap replicates. The analyses show that *Cyanophora paradoxa*, *Glaucocystis nostochinearum*, and *Gloeochaete wittrockiana* form an evolutionarily distinct photosynthetic lineage that forms a sister group to the cryptomonad algae. The bootstrap analyses support the monophyly of these Glaucocystophyta, as does the relatively long common branch length that unites them in the maximum-likelihood phylogeny. It should be stated that though the bootstrap is the most widely used statistical method for assessing the stability of topological
FIG. 1.—Phylogeny of eukaryotes based on small-subunit rRNA sequence comparisons inferred with the maximum-likelihood method (fastDNAm, V 1.0; Olsen et al. 1994) using 1,621 unambiguously aligned nucleotides. This phylogeny is rooted within the branch leading to Dictyostelium discoideum. The bootstrap values above the internal nodes are inferred from a distance analysis of the same data set using a Kimura (1980) matrix as input for a neighbor-joining phylogenetic reconstruction (Saitou and Nei 1987). The bootstrap values shown below the internal nodes in italicized script are inferred from a weighted maximum-parsimony analysis (PAUP V3.1.1; Swofford 1993). The maximum-parsimony analysis resulted in a single-most parsimonious phylogeny with a consistency index of 0.607. Only bootstrap values above 60% (except for the node defining the cryptophyte/glaucocystophyte lineage [shown as a thicker line]) are recorded. The G+C contents of the aligned sequence positions of the taxa used in the phylogenetic analyses ranged from 43% to 53%.

elements in phylogenies, this estimate seems to be a conservative measure of topology (Zharkikh and Li 1992). Computer simulations have shown that values as low as 70% can define known correct topologies (for discussion, see Hillis and Bull 1993; Wainright et al. 1993). For this reason, bootstrap values over 70% (Hillis and Bull 1993) likely define stable groupings, though these values should never be taken as an ultimate measure of evolutionary relatedness.

Our analyses do not position G. vacuolata within the Glaucocystophyta but instead show that this species groups with the red algae (Rhodophyta). This is an important result since G. vacuolata has been hypothesized to be an evolutionary "link" between the glaucocystophytes and the red algae on the basis of plastid characters (i.e., with loss of the peptidoglycan wall in the G. vacuolata plastid; Cavalier-Smith 1982, 1987); rhodoplasts also have two envelope membranes and contain phycobilisomes and unstacked thylakoids. The positioning of G. vacuolata in figure 1 as a member of the red algae is supported, however, by other morphological and biochemical data (e.g., presence of R-phycocyanin in the G. vacuolata plastid [the glaucocystophyte cyanelles contain C-phycocyanin; Richardson and Brown 1970] and the complete absence of flagella and basal bodies). Further, McCracken et al. (1980) have argued, based on electron microscope observations, that the multiple "cyanelles" of G. vacuolata are in fact a single highly lobed rhodoplast. These authors suggested placing G. vacuolata in the red algal order Porphyridiales. This re-
The phylogenetic analyses also provide some insights into the possible origin of plastids with four bounding membranes; these plastids are presumably derived from the secondary endosymbiosis(es) of a eukaryotic alga (Gibbs 1981; Cavalier-Smith 1982) and with the prasinophyte green algae on the basis of the common possession of a cruciate flagellar root system with associated multilayered structures (MLSs) and similarities in the flagellar development cycle (Melkonian 1983; Heimann et al. 1989). The user-defined tree analyses (table 1) support the monophyletic origin of the glaucocystophyte/cryptophyte (i.e., the “best” tree shown in fig. 1) since the disruption of this clade resulted in a significantly “worse” tree, as did the positioning of the glaucocystophytes with either the red or green algae. Though not conclusive, these data provide support for the topology presented in figure 1. The phylogenetic analyses do not show a close evolutionary relationship between the glaucocystophytes and red and green algae and therefore do not resolve the origin of their plastids (characterized by two envelope membranes). It is possible that cyanelles and red and green algal plastids have independent endosymbiotic origins; however, the bulk of sequence, biochemical, and ultrastructural data favor a single monophyletic origin of these organelles (Douglas and Turner 1991; Helmhchen et al., in press). The inability to show a monophyletic origin of the host cells that contain these plastids may reflect the limited resolution of rRNA comparisons in the region of the phylogeny characterized by the radiation of these photosynthetic lineages.

Table 1
Results of User-Defined Tree Analyses Using the Maximum-Likelihood Method

<table>
<thead>
<tr>
<th>Tree</th>
<th>Log-Likelihood</th>
<th>Difference in Log-Likelihood</th>
<th>Significantly Worse?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Best tree</td>
<td>-30,289.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Independent</td>
<td>-30,308.95</td>
<td>-19.73</td>
<td>Yes</td>
</tr>
<tr>
<td>With rhodophytes</td>
<td>-30,372.53</td>
<td>-83.31</td>
<td>Yes</td>
</tr>
<tr>
<td>With chlorophytes</td>
<td>-30,319.66</td>
<td>-30.44</td>
<td>Yes</td>
</tr>
</tbody>
</table>
are found in cryptophytes, chlorarachniophytes, heterokonts, and prymnesiophytes. One hypothesis proposes a single secondary endosymbiotic origin of plastids with four bounding membranes within the common ancestor of a monophyletic “host” cell assemblage defined by the cryptophytes, chlorarachniophytes, heterokonts, and prymnesiophytes (Cavalier-Smith 1993). The phylogeny shown in figure 1 is not consistent with this hypothesis. The Chlorarachniophyta share a closer evolutionary relationship with filose amoebae (i.e., *P. chromatophora*, *Euglypha rotunda*; Bhattacharya et al., in press) than with cryptophytes, heterokonts, or prymnesiophytes. The divergence of the photosynthetic heterokont algae is preceded by the independent divergences of the plastidial taxa, *Labyrinthuloides minuta* and *Cafeteria roenbergensis* (Leipe et al. 1994). Further, the plastidial *Goniomonas truncata* diverges before the photosynthetic members of the cryptophyte lineage (McFadden et al. 1994). Barring multiple, independent losses of the plastid and the entire chloroplast endoplasmic reticulum complex (typical of plastids with four membranes) in the filose amoebae and earlier-diverging, plastidial heterokonts, and *G. truncata*, these data are more easily explained by independent secondary endosymbioses of eukaryotic algae giving rise to plastids in the chlorarachniophytes (Bhattacharya et al., in press), photosynthetic heterokonts (Leipe et al. 1994), and photosynthetic cryptophytes (McFadden et al. 1994). Sequence data are required from the cyanelle of *P. chromatophora* to determine whether its origin (primary or secondary endosymbiosis) is independent from that of the cyanelle of the glaucocystophytes from which it is evolutionarily distantly related (see Bhattacharya et al., in press, for discussion).

In conclusion, our results provide further evidence for the evolutionary diversity of photosynthetic protists and suggest that glaucocystophytes and cryptophytes share a common ancestry. The paucity of biochemical and genetic data precludes a deeper understanding of the biological relationship between these two distinctive protistan lineages. Further, we speculate that there have been, minimally, three independent eukaryotic secondary endosymbioses giving rise to plastids of chlorarachniophytes, photosynthetic heterokonts, and photosynthetic cryptophytes within the crown group radiation. Phylogenies based on plastid characters may provide, therefore, greater insight into the lateral transfer of these organelles (particularly via secondary endosymbioses) rather than into the evolutionary history of the host cells that contain them.

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