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Plastid-Targeting Peptides from the Chlorarachniophyte *Bigelowiella natans*

MATTHEW B. ROGERS,[a] JOHN M. ARCHIBALD,[a,1] MATTHEW A. FIELD,[a] CATHERINE LI,[b] BORIS STRIEPEN[b] and PATRICK J. KEELING[a]

[a]*Canadian Institute for Advanced Research, Department of Botany, University of British Columbia, 3529-6270 University Boulevard, Vancouver, BC, V6T 1Z4, Canada, and*

[b]*Center for Tropical & Emerging Global Diseases & Department of Cellular Biology, University of Georgia, 724 Biological Sciences Building, Athens, Georgia 30602, USA*

ABSTRACT. Chlorarachniophytes are marine amoeboflagellate protists that have acquired their plastid (chloroplast) through secondary endosymbiosis with a green alga. Like other algae, most of the proteins necessary for plastid function are encoded in the nuclear genome of the secondary host. These proteins are targeted to the organelle using a bipartite leader sequence consisting of a signal peptide (allowing entry in to the endomembrane system) and a chloroplast transit peptide (for transport across the chloroplast envelope membranes). We have examined the leader sequences from 45 full-length predicted plastid-targeted proteins from the chlorarachniophyte *Bigelowiella natans* with the goal of understanding important features of these sequences and possible conserved motifs. The chemical characteristics of these sequences were compared with a set of 10 *B. natans* endomembrane-targeted proteins and 38 cytosolic or nuclear proteins, which show that the signal peptides are similar to those of most other eukaryotes, while the transit peptides differ from those of other algae in some characteristics. Consistent with this, the leader sequence from one *B. natans* protein was tested for function in the apicomplexan parasite, *Toxoplasma gondii*, and shown to direct the secretion of the protein.

Key Words. Secondary endosymbiosis, signal peptide, transit peptide.

PLASTIDS, the light-harvesting organelles of plants and algae, are the product of an ancient symbiosis between a cyanobacterium and a non-photosynthetic eukaryote. This process is referred to as primary endosymbiosis, and has given rise to the plastids of green algae and land plants, red algae and glaucocystophytes. The primary plastids of red and green algae have also spread laterally amongst unrelated eukaryotes by a process called secondary endosymbiosis, in which a primary plastid-containing alga is engulfed and retained by a non-photosynthetic eukaryote (Archibald and Keeling 2002). Secondary plastid-containing organisms account for a significant fraction of present-day algal diversity: secondary algae are abundant, genetically diverse and contain a variety of plastid types. Secondary plastid-containing lineages include the haptophytes, heterokonts, cryptomonads, dinoflagellates, and apicomplexan parasites, which all contain red algal endosymbionts, as well as the euglenids and chlorarachniophytes, which contain green algal endosymbionts.

Chlorarachniophytes are unicellular, amoeboflagellate algae found in marine environments that have acquired a plastid through secondary endosymbiotic uptake of a green alga (Hibberd and Norris 1984; Ludwig and Gibbs 1989; McFadden et al. 1994). As a consequence, the plastids of chlorarachniophytes are bounded by four membranes: the inner-two membranes are homologous to those of cyanobacteria and the primary plastids of green algae and plants, and the outer two are derived from the plasma membrane of the green algal endosymbiont and the secondary host endomembrane system, respectively (McFadden 2001). As is the case in the red algal symbiont of cryptomonads, the chlorarachniophyte endosymbiont retains a highly reduced algal nucleus, or ''nucleomorph'' (Hibberd and Norris 1984; Ludwig and Gibbs 1989; McFadden et al. 1994), nested between the second and third plastid membranes (i.e. in the residual cytosol of the endosymbiont).

The process of secondary endosymbiosis has important ramifications for protein targeting in secondary plastid-containing algae. This is because plastid genomes encode only a small fraction of the genes necessary to encode all plastid proteins. Most of these proteins are encoded by nuclear genes and the products are post-translationally targeted to the plastid using an amino-terminal extension called a transit peptide (McFadden 1999). Accordingly, most genes for plastid proteins in chlorarachniophytes would have been encoded in the nuclear genome of the green algal endosymbiont. However, preliminary sequencing of the nucleomorph genome shows that few such genes remain (Gilson and McFadden 1996): in the course of its secondary endosymbiotic integration, most of these genes were once again transferred, this time to the nuclear genome of the secondary host (Archibald et al. 2003; Deane et al. 2000). The protein products of these genes, therefore, must be targeted across four membranes to the plastid stroma (and in some instances across a fifth, thylakoid membrane). In all secondary plastids examined so far, this process involves the addition of a second N-terminal extension (for review see McFadden 1999). First, the protein is targeted to the host endomembrane system using a signal peptide, which directs the co-translational import of precursor proteins to the endoplasmic reticulum and is subsequently cleaved off (Blobel and Dobberstein 1975a, b). Second, precursor proteins are imported across the inner and outer chloroplast envelope following the general import pathway involving interaction of a transit-peptide with the plastid envelope and TOC and TIC complexes, common to the primary plastids of glaucocystophytes, red algae, green algae and plants (Bruce 2001; Schleiff and Soll 2000). How proteins are specifically directed to the plastid once they are in the host endomembrane system, and how proteins cross the second membrane from the outside (homologous to the algal cytoplasmic membrane, which has been lost in euglenids and dinoflagellates) are both unknown, and represent two of the outstanding mysteries in this process (McFadden 1999; van Dooren et al. 2001).

In cryptomonad, heterokont and haptophyte algae (chromists), the outer-most membrane of the plastids are continuous with the endoplasmic reticulum (ER) (Gibbs 1981; Ishida et al. 2000), so plastid-targeted proteins are either co-translationally inserted directly into the compartment where the plastid endosymbiont resides (Gibbs 1979), or enter the ER and are translocated across the outer membrane through lumenal connections, as has been demonstrated in heterokonts with smooth outer membranes (Ishida et al. 2000). In contrast, the outermost membrane of the three membrane secondary plastids of dinoflagellates and euglenids are not contiguous with the host endomembrane, and these organisms use an elaborate system of vesicles to specifically target proteins to the plastid using the Golgi apparatus (Nassoury et al. 2003; Sulli et al. 1999; Sulli

Corresponding Author: P. Keeling—Telephone number: +1 604 822 4906; Fax number: +1 604 822 6089; E-mail: pkeeling@interchange.ubc.ca

[1] Present address: Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia, B3H 1X5 Canada.

and Schwartzbach 1995, 1996). Interestingly, these proteins are only partially translocated across the ER membrane, so the mature peptide rests on the cytosolic face of the Golgi vesicles. The outer-most membrane of chlorarachniophyte and apicomplexan plastids is also not detectably contiguous with the host endomembrane, but the route taken by plastid proteins in these organisms is not known, although it has been proposed that they may travel through the Golgi (Bodyl 1997; Waller et al. 2000). Given that no other group is known to direct proteins to their plastids through the lumen of the Golgi, this route may be more difficult than previously conceived.

As a first step in characterising the route traveled by nuclear-encoded, plastid-targeted proteins in chlorarachniophytes, we have analysed plastid-targeting leader sequences from 45 plastid-targeted proteins from *Bigelowiella natans*, compared the N-termini of these proteins to 10 ER-targeted and 38 cytosolic *B. natans* proteins, and tested the heterologous function of one leader in the apicomplexan, *Toxoplasma gondii*. Overall, the characteristics of *B. natans* signal peptides and some characteristics of transit peptides are similar to those found inother well-studied systems, while other features of transit peptides are different than those found in other algal groups.

MATERIALS AND METHODS

Assembling a data set of cytosolic, ER, and plastid proteins from *B. natans*. Seventy-eight predicted plastid-targeted proteins were identified from a *B. natans* EST project by Archibald et al. (2003), and an additional gene encoding a full-length plastid-targeted protein identified as a phosphoglycolate phosphatase precursor was added to this dataset. Of these 79 proteins, we have identified 45 clearly full-length transcripts with N-terminal extensions predicted to encode signal peptides (Genbank accession numbers: AAO89070, AAP79136, AAP79140–AAP79142, AAP79144, AAP79147–AAP79150, AAP79152, AAP79153, AAP79155–AAP79161, AAP79164, AAP79166, AAP79167, AAP79170, AAP79174, AAP79175, AAP79177, AAP79179, AAP79181, AAP79183, AAP79187–AAP79189, AAP79192–AAP79196, AAP79199, AAP79203, AAP79208–AAP79211, AAP79216, AY611522.) cDNAs encoding putative plastid-targeted proteins were identified based on their phylogenetic relationship to plastid-targeted genes in other organisms, their homology with proteins involved in pathways specific to plastids, and their possession of a bipartite leader sequence consisting of a signal and transit peptide (Archibald et al. 2003). Three genes that encoded substantial N-terminal leaders but lacked obvious signal peptides were excluded from further analysis. Ten ESTs for full-length genes encoding proteins known in other systems to reside within the endomembrane system or to be secreted were identified, and each of these was completely sequenced. Thirty-eight ESTs encoding full-length transcripts of cytosolic proteins were also identified in the same way, and each completely sequenced (Table I). The 49 newly sequenced genes were submitted to GenBank as Accessions AY542966–AY543013, AY611522.

Signal peptide predictions and analysis. The N-termini of the plastid-targeted proteins were first analysed using the neural network prediction server SIGNALP v. 2.0 (Nielsen et al. 1997) to examine the first 50 amino acids for putative signal peptides. Three proteins were identified as having large N-terminal leaders that included a methionine codon, but were not predicted to encode a signal peptide and did not encode a stop codon upstream of the potential start codon. These proteins were excluded from further analysis due to the possibility that their leaders may not be completely sequenced. All signal and transit peptides were aligned according to their predicted cleavage sites. To verify these predictions and demonstrate any deviations from the expected chemical properties of these sequences, the overall characteristics of regions upstream and downstream of the predicted cleavage site were examined according to several criteria. Perl scripts were written to generate sliding-window Kyte-Doolittle hydropathy profiles (Kyte and Doolittle 1982) and amino acid frequency profiles for the 15 amino acids upstream and 20 amino acids downstream of the predicted signal cleavage site using a five amino acid window. Amino acid frequencies were divided in to three categories representing properties known to be abundant or depleted in the signal and transit peptides in other organisms: hydroxylated (ST), basic (HKR) and acidic (DE) residues. The ten non-plastid, endomembrane-targeted proteins were analysed in the same way to serve as a positive control.

Table **1**. New cytosolic, nuclear and endomembrane-targeted proteins in *Bigelowiella natans.*

Cytosolic & nuclear
Cofilin
ADP ribosylation factor
Histone H2B
RAS related GTP binding protein
Proteasome beta-subunit
Calmodulin
Actin depolymerizing factor
Transcription factor BTF3
Small nuclear ribonucleoprotein SM-D1
Clathrin assembly protein
Roadblock
Peroxidase
Glutathione-S-transferase
Actin related protein
Ubiquitin conjugating protein
Mago nashi homologue
Splicing factor
Calmodulin like myosin chain
RAB2
Small nuclear ribonucleoprotein
U6 snRNA associated SM like protein
F-actin capping protein beta subunit
Coronin
RAS-like GTPase
Ubiquitin conjugating enzyme E2-1
Ubiquitin conjugating enzyme E2-2
ADP ribosylation factor 1
Dynein 8 kDa light chain
RNA polymerase 2 15.9 kDa subunit
Selenoprotein W
Guanine nucleotide binding protein
Alpha tubulin 1
Alpha tubulin 2
Dynein outer arm light chain
U1 small nuclear ribonucleoprotein
Translation initiation factor 5A
Translation initiation factor 6
GTP binding nuclear protein RAN
Endomembrane-targeted
Calreticulin
Cathepsin Z
COP protein
Cyclophilin B
Cystein proteinase
Digestive cysteine proteinase
Folate receptor homologue
KDEL receptor
Protein disulfide isomerase
Serine threonine proteinase

Transit peptide prediction and analysis. Transit peptides were analyzed in a similar fashion to signal peptides, however transit peptide cleavage sites could not be assigned as reliably. To determine the approximate location of a potential cleavage site, the chloroplast transit peptide prediction server ChloroP (Emanuelsson et al. 1999) was used in combination with a multiple alignment of homologous proteins from other eukaryotes and eubacteria. In instances where ChloroP was unable to predict a transit peptide, or if the cleavage site of the transit peptide fell within the conserved portion of the mature sequence (as indicated by the alignment), or if the transit peptide was predicted to be much shorter than the remaining leader sequence, the end of the transit peptide was estimated based on the position in the alignment corresponding to the start of a cytosolic homologue in other eukaryotes or eubacteria. For this reason, putative transit peptide cleavage sites represent only a rough estimate of the end of the transit peptide. Using the putative cleavage site as a point of reference for all transit peptides, 20 amino acids upstream and 20 amino acids downstream were analyzed for hydropathy and amino acid frequencies using the same approach as described for signal peptides. The overall amino acid composition of transit peptides was also analysed by concatenating the 45 inferred transit peptides and comparing their amino acid frequencies with those of 45 concatenated mature plastid-targeted proteins and the 38 concatenated cytosolic proteins.

Heterologous activity of *B. natans* plastid-targeting leader in *T. gondii*. To test heterologous expression in *T. gondii* the coding sequence of *B. natans* ribulose 1,5-bisphosphate carboxylase (RuBisCO) was introduced into a parasite expression vector by recombination cloning. The sequence was amplified from cDNA clone by PCR using gene specific primers introducing half of the required attB recombination sites (5′-AAAAAGCAGGCTAAAATGATGAGAAACGTTGCCCT 5′-AGAAAGCTGGGTACCAGGAGTAAGTGAATCCTCC) for 10 cycles. An aliquot of this reaction was used as template in a second reaction using and excess of universal attB primers and 5 low and 20 high stringency cycles (5′-GGGGACAAGT TTGTACAAAAAAGCAGGCT, 5′-GGGGACCACTTTGTAC AAGAAAGCTGGGT, see (Gubbels et al. 2003) for additional detail). The PCR product was cloned using a one tube BP/LR recombinase reaction using topoisomerase I treated destYFP/sagCAT destination vector (Striepen et al. 2002). The resulting plasmid RuBisCO-YFP, places the RuBisCO coding sequence under the control of the constitutive *T. gondii* alpha-tubulin promoter and in translational fusion upstream of yellow fluorescent protein.

RH-strain *T. gondii* tachyzoites were passaged in confluent human foreskin fibroblasts (HFF) and transfected essentially as described previously (Striepen et al. 2002). In brief, 10^7 freshly harvested tachyzoites were resuspended in 300 μl cytomix and mixed with 50-μg plasmid DNA in 100 μl cytomix. Electroporation was performed using a BTX ECM 630 electroporator (Genetronics, San Diego, CA) set at 1,500 kV, 25 Ω and 25 μF in a 2-mm cuvette. Parasites were inoculated into coverslip cultures and observed using a DM IRB inverted microscope (Leica, Wetzlar, Germany) equipped with a 100 Watt HBO lamp 24 h after transfection. YFP and RFP expression was detected using appropriate filter sets (460/40 nm bp, em 527/30 nm bp, and ex 515/45 nm bp, em 590 nm lp, respectively). Images were recorded using a digital cooled CCD camera (Hamamatsu, Bridgewater, NJ) and processed and analyzed using Openlab software (Improvision, Quincy, MA).

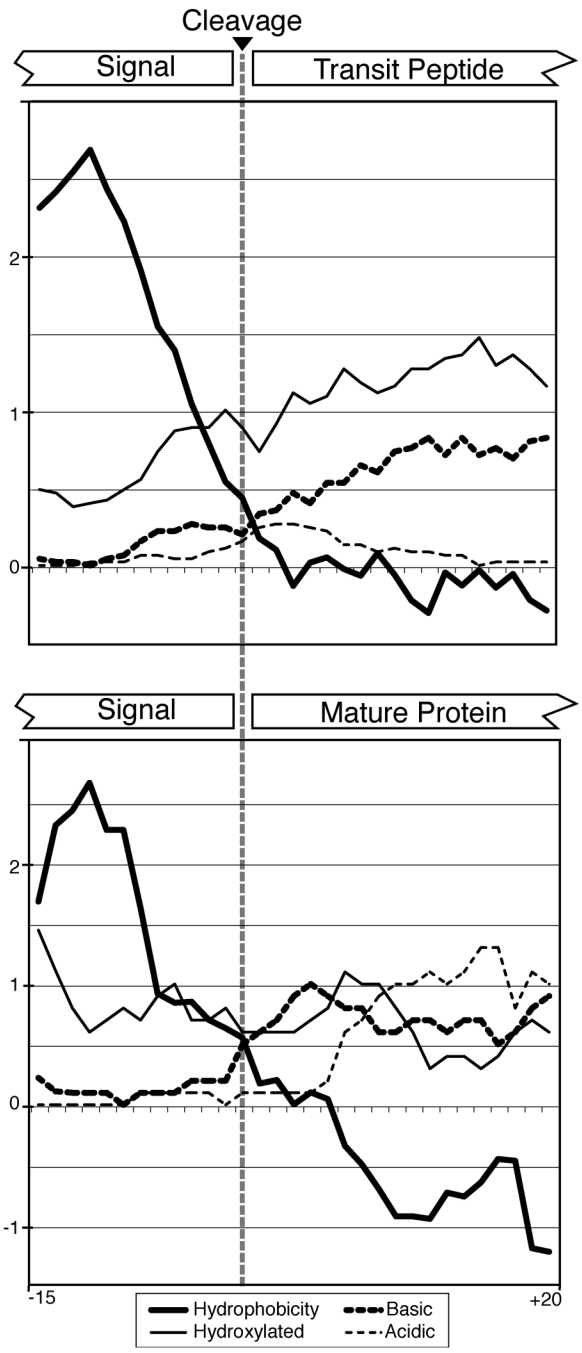

Fig. **1**. Properties of the signal peptide–transit peptide boundary of 45 *Bigelowiella natans* plastid-targeted proteins (top) compared to the signal peptide–mature protein boundary of 10 ER-targeted proteins (bottom). The X-axis corresponds to the position of the sliding window, while the Y-axis corresponds to Kyte-Doolittle values for hydrophobicity or the number of hydroxylated (ST), basic (HKR), or acidic (DE) amino acids per window divided by the size of the window respectively. All proteins are aligned on the predicted cleavage site, which is indicated by a dashed grey line.

RESULTS AND DISCUSSION

***B. natans* signal peptides.** Forty-five inferred plastid-targeted proteins from *B. natans* were predicted to encode N-terminal signal peptides when examined using SignalP. The predicted signal peptides varied in length from 16–47 residues, with a median length of 33 amino acids. Forty-four percent of the signal peptides examined (20 out of 45) possessed a motif consisting of a serine residue, followed by three neutral or hydro-

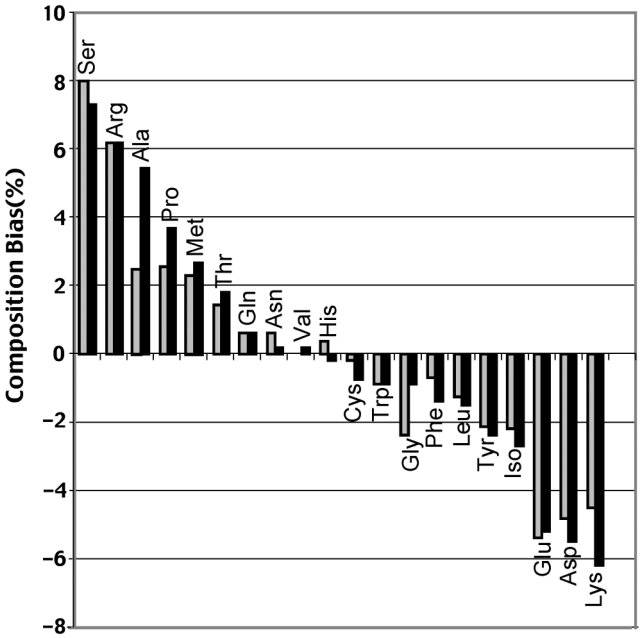

Fig. **2**. Biased amino acid composition of transit peptides in *Bigelowiella natans*. Bars indicate the difference in percent amino acid composition between 45 concatenated transit peptides compared with the concatenated mature plastid proteins (grey bars) and 38 concatenated cytosolic proteins (black bars). Bars above the X-axis indicate an overabundance of that amino acid while bars below the X-axis indicate a depletion of that amino acid.

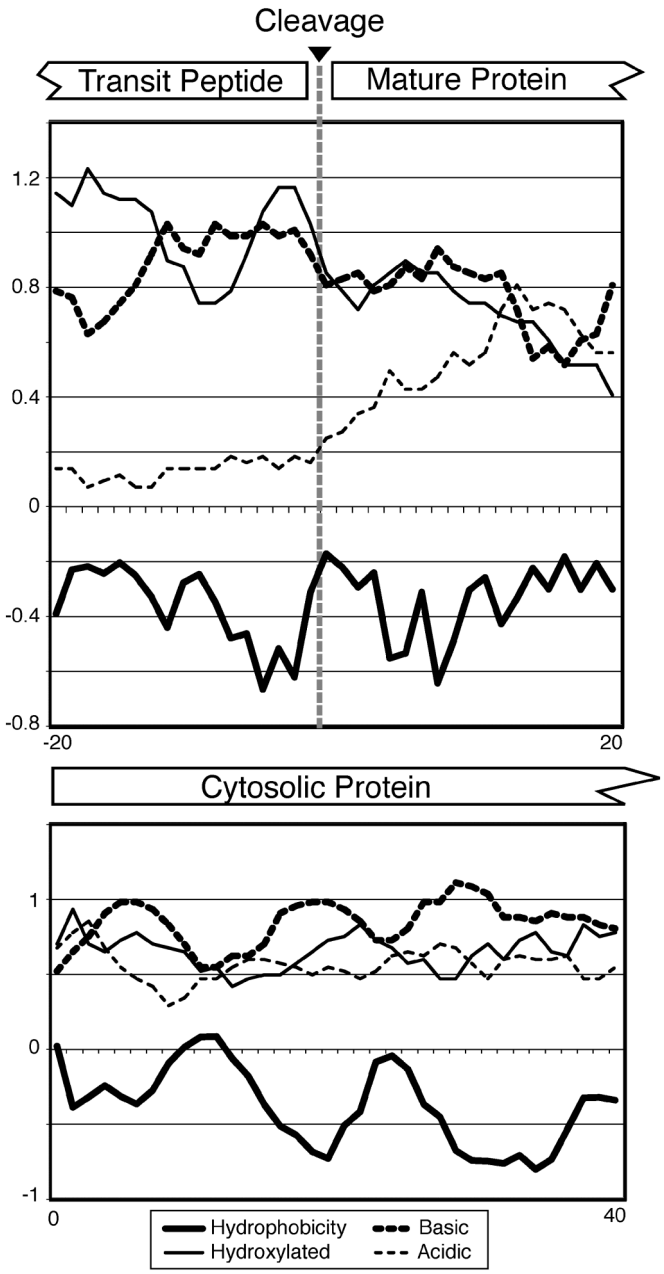

Fig. **3**. Properties of the transit peptide–mature protein boundary of 45 plastid-targeted *Bigelowiella natans* proteins (top) compared with the amino terminal region of 38 cytosolic proteins. Axes and lines are as in Fig. 1.

phobic amino acids, and an asparagine between 3 and 25 amino acids upstream of the cleavage site. Asparagines are uncommon in most signal peptides (Hoyt and Gierasch 1991) making the parallel occurrence of this motif unlikely. While the appearance of this motif in nearly half of the signal peptides examined is interesting, no role for this motif is obvious. This motif was absent in the signal peptides of predicted endomembrane resident and secreted proteins. The significance of this observation is unknown.

The predicted cleavage sites of *B. natans* signal peptides corresponded to a von-Heijne motif (von Heijne 1983, 1984) with the −1 position occupied by an alanine residue in roughly 45% (21 out of 45) of the plastid targeted proteins examined, and by a glycine, serine or cysteine in all other cases. The −3 position is typically occupied by a variety of small uncharged amino acids such as alanine, valine, leucine, serine or threonine. The overall chemical properties surrounding the predicted cleavage sites of these 45 peptides were examined and compared with the equivalent region of proteins known to be targeted to the endomembrane system (Fig. 1). In other systems that have been examined, signal peptides are generally rich in hydrophobic residues and small neutral residues, but depleted of acidic residues (Nielsen et al. 1997). The signal peptides of plastid-targeted proteins in *B. natans* conform to these general expectations: alanine was the most abundant amino acid (not shown), while the acidic residues aspartic acid and glutamic acid were depleted (Fig. 1, upper). These trends were also reflected in the signal peptides of *B. natans* ER-targeted proteins (Fig. 1, lower). Overall, the signal peptides of plastid-targeted proteins of *B. natans* appear to be chemically similar to those of secreted or endomembrane resident proteins, suggesting that plastid proteins are not likely distinguished by a specific type of signal peptide that directs plastid-targeted proteins to the appropriate compartment. Although signal peptides from some plastid-targeted proteins possess a distinct motif absent in the signal peptides of endomembrane resident or secreted proteins, no clear role for this motif is apparent.

***B. natans* transit peptides.** Transit peptides are typically more difficult to predict than signal peptides, but are generally variable in length; plant transit peptides are typically rich in serine, threonine and arginine residues, and depleted in acidic amino acids (Keegstra et al. 1989). The inferred transit peptides of *B. natans* were also highly variable in length, ranging between 20 and 83 amino acids. The overall amino acid frequency of these peptides was compared with that of mature plastid

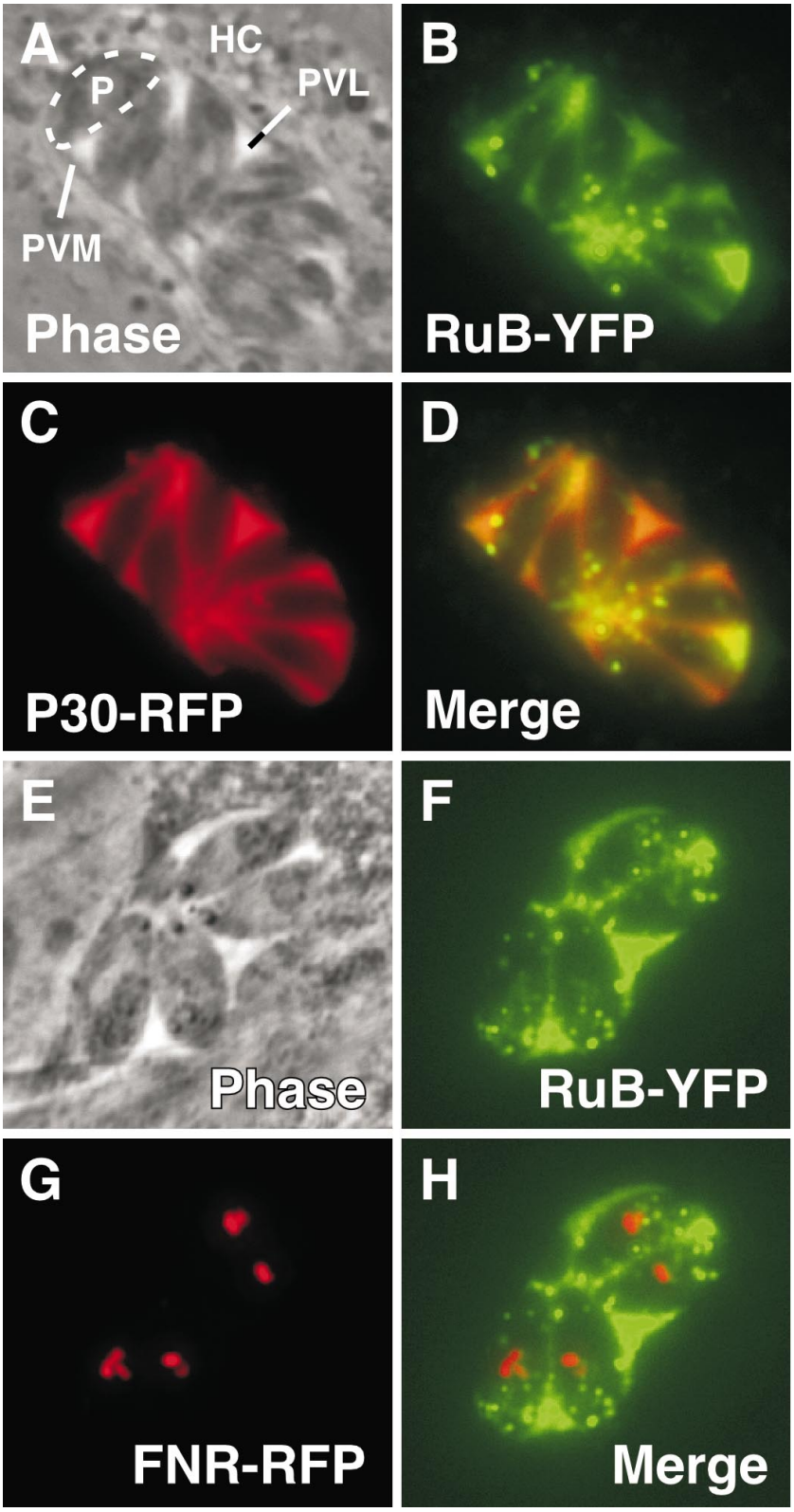

Fig. **4**. Heterologous expression of the *Bigelowiella natans* RuBisCO small subunit leader peptide in the apicomplexan *Toxoplasma gondii*. **A–D**: co-expression of RuBisCO-YFP with secreted protein P30. Host cells were infected with *T. gondii* co-expressing RuBisCO-YFP fusion protein and P30-RFP or FNR-RFP fusion proteins respectively. (**A**) Phase contrast image of host cell (HC) showing a parasitophorous vacuole, the lumen of which (PVL) contains eight parasites (P). (**B**) RuBisCO-YFP fusion protein localizes to the parasitophorous vacuole (diffuse fluorescence around parasites) and dense granules (punctate fluorescence in parasites), both indicative of secretion. (**C**) The distribution of the established secretion marker P30-RFP is the same as that of RuBisCO-fusion protein, and the two show strong co-localization when the green and red channel are merged (**D**). This suggests that the RuBisCO leader directs the secretion of the protein. **D–H**: co-expression of RuBisCO-YFP with plastid-targeted protein FNR. (**E**) Phase contrast image of host cell and parasitophorous vacuole. (**F**) Expression of RuBisCO-YFP in same parasites. (**G**) The plastid in *T. gondii* is a single round organelle localized apical of the nucleus, which has been labeled by stable expression of FNR-RFP (Striepen et al. 2000). (**H**) Expression of RuBisCO-YFP in this background shows no co-localization (merged green and red channel).

proteins and cytosolic proteins, and found to follow many of the expected trends (Fig. 2). In particular, serine and arginine residues are enriched when compared with both mature plastid-targeted proteins and cytosolic proteins, while glutamic acid and aspartic acid are depleted. Interestingly, the positively charged amino acid lysine was the most depleted compared with both mature plastid-targeted proteins and cytosolic proteins, while this is the most abundant amino acid in the transit

peptides of the malaria parasite, *Plasmodium falciparum* (Foth et al. 2003). This is interpreted as depletion since the frequency of lysine is lower in transit peptides than in both cytosolic and mature plastid proteins. In general, the mature plastid-targeted and cytosolic proteins shared similar amino acid frequencies, with the exception of alanine, which is more highly represented in cytosolic proteins (Fig. 2). Altogether, the overall amino acid content of *B. natans* transit peptides is within the bounds of expected properties, although the apparently extreme depletion of lysine does distinguish these sequences from other transit peptides.

The chemical properties of the region surrounding the inferred transit-peptide cleavage site were also analysed and compared with the N-termini of cytosolic proteins (Fig. 3). In general, the region upstream of the cleavage site was slightly enriched in hydroxylated and basic residues (Fig. 3, upper), a trend that is also noticeable at the signal-transit boundary (Fig. 1, upper). Basic amino acids, in particular arginine, are concentrated near the predicted cleavage site of the transit peptide and less prominent near the N-terminus, which has also been reported for plant transit peptides (Claros et al. 1997). Conversely, the frequency of acidic residues increases at the transit peptide-mature protein boundary, so that the overall basic nature of the transit peptides is more the result of a depletion of acidic residues than an overrepresentation of basic ones. These characteristics are also found in plant transit peptides, where they are thought to assist the plastid transit peptide in interacting with the negatively charged outer membrane of the plastid (Bruce 2001) as well as potentially serving as a charged binding site for processing peptidases (Richter and Lamppa 2002). These results are consistent with previous descriptions of the signal and transit peptides of the LHCII proteins in *B. natans* (Deane et al. 2000). In general, the transit peptides of *B. natans*, are enriched in the hydroxylated amino acid serine, the basic residue arginine, and are depleted of acidic residues and lysine.

Heterologous targeting in *T. gondii*. Analyses of targeting peptide primary structure are often good guides for experimental design (Foth et al. 2003), but no transformation system exists for a chlorarachniophyte. Instead, we have tested one *B. natans* signal and transit peptide in the apicomplexan parasite *Toxoplasma gondii*. Because of their medical importance, the plastids of the apicomplexan intracellular parasites (apicoplasts) now rank among the best studied of any algal group, despite being the most recently discovered. Plastid targeting in *P. falciparum* and *T. gondii* have been examined in some detail, as have the characteristics of plastid-targeting leader sequences from *Plasmodium* (DeRocher et al. 2000; Foth et al. 2003; Roos et al. 1999; Waller et al. 1998; Waller et al. 2000; Yung and Lang-Unnasch 1999; Yung et al. 2001; Yung et al. 2003). In general, the apicomplexan signal peptides exhibit chemical characteristics typical of signal peptides in other organisms, but the transit peptides exhibit a number of unexpected properties. The transit peptides of *Plasmodium* in particular are highly enriched in lysine and asparagine residues (Foth et al. 2003). Currently, only four transit peptides have been described in detail from *Toxoplasma gondii*, these transit peptides are similar to plant transit peptides in being rich in hydroxylated and basic amino acids (DeRocher et al. 2000). Such characteristics are shared with the transit peptides of *B. natans*. The predicted *B. natans* RuBisCO transit peptide is similarly rich in hydroxylated residues, but with a lower frequency of arginines than that seen in other predicted targeting peptides.

Despite their divergent properties, the transit peptides of *Toxoplasma* and *Plasmodium* are reported to be interchangeable, and plant transit peptides are also reported to function in *Plasmodium* (Roos et al. 1999; Waller et al. 2000), although these observations need to be followed up with further experimentation. To test whether one *B. natans* signal and transit peptide possesses the biochemical characteristics necessary to function in a heterologous system featuring secondary plastids, transfection experiments in *T. gondii* were performed. This is not intended to be a comprehensive test of the interchangability of these sequences, but rather a preliminary test of our observations on the nature of *B. natans* peptides. Cells were transfected with a construct encoding YFP fused to the C-terminus of the leader from the *B. natans* RuBisCO small subunit protein. In the resulting transfectants (Fig. 4B), YFP localizes to the parasitophorous vacuole, with some labeling of dense granules. When cotransfected with the secretory marker P30-RFP (Striepen et al. 2001), YFP and RFP co-localise (Fig. 4D), indicating that the RuBisCO-YFP fusion protein is secreted, and not targeted to the plastid. Transfection of RuBisCO-YFP into a parasite line stably expressing plastid targeted FNR-RFP (Striepen et al. 2000) further confirms this observation, there is no overlap between FNR-RFP and RuBisCO-YFP labeling (Fig. 4H). In the case of the RuBisCO leader, therefore, the predictions based on sequence characteristics are met, namely that the *B. natans* signal peptide is able to function in *T. gondii* while the chemical characteristics of the *B. natans* transit peptide are insufficient for targeting to the apicomplexan plastid. Though this may reflect the inability of *Toxoplasma* signal peptidase enzymes to recognize and cleave signal peptides specific to *B. natans*, thus preventing interactions between the transit peptide and apicoplast, it seems unlikely that such unprocessed proteins would be secreted into the parasitophorous vacuole. Alternatively, the inability of this *Bigelowiella* transit peptide to function in *Toxoplasma* may represent a fundamental difference between the architecture of transit peptide recognition and plastid import in these two distantly related systems. Now that a significant number of *B. natans* plastid-targeting leaders are known, it would be interesting to test all such sequences for activity in apicomplexa to see if the RuBisCO example is representative of *B. natans* transit peptides in general, or if other chlorarachniophyte plastid-targeting sequences are sufficient to direct proteins in to the apicoplast. Further examination of complexes involved in protein translocation across the plastid envelope membranes in these two organisms may also shed some light on potential differences in plastid import. If the RuBisCO transit peptide is representative of typical *B. natans* plastid-targeting leaders, additional experiments would also be required to determine which characteristics are most critical to the function of *B. natans* targeting peptides, and which distinguish them from the targeting peptides of other organisms. The results presented here suggest that such features will more likely be specific to transit peptides than signal peptides, which appear to be universal amongst secondary plastid-containing algae.

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