evolution via the replacement of two-membrane plastids would have to be accompanied by the origin of a new mechanism of protein targeting to the eukaryotic endosymbiont. The process would be very complicated, as it would require (1) the acquisition of signal peptides by the hundreds of nuclear-encoded plastid proteins of the host and (2) the evolution of a mechanism sorting these proteins inside the *trans*-Golgi network into the proper transport vesicles[5]. Moreover, the latter innovation would be associated with the acquisition of a new Golgi-sorting signal by the host proteins.

Interestingly, all these difficulties can be easily overcome by assuming that four-membrane plastids have evolved via the replacement of three-membrane plastids[5]. As with four-membrane plastids, protein import into three-membrane plastids proceeds through both the ER and Golgi apparatus, and their nuclear-encoded proteins have a signal peptide[11]. As the outermost membrane in each of these plastids is derived from the phagosomal membrane[5,12], the targeting of the host plastid proteins to the eukaryotic endosymbiont would probably require only changes in SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins[5]. Although, according to the commonly accepted view, three-membrane plastids have originated from eukaryotic algae[13], numerous data indicate that their ancestors were phototrophic prokaryotes[12]. Thus, the evolutionary pathway of four-membrane plastids would be an example of a secondary (replacement of a prokaryotic by a eukaryotic plastid) endosymbiosis rather than a tertiary (replacement of a eukaryotic plastid by another eukaryotic plastid) endosymbiosis.

Dinoflagellates are an excellent taxon to study secondary endosymbiosis. Contrary to other phototrophic eukaryotes, these protists have acquired a bewildering array of plastids from various eukaryotic algae, such as green algae, cryptophytes and heterokonts[14,15]. These 'unusual' plastids represent various stages of reduction of the eukaryotic endosymbiont, and their envelopes consist of two to five membranes[14,15]. As the ancestral plastid in dinoflagellates is a three-membrane plastid containing peridinin, all kinds of unusual plastids should be presumed to have evolved via the replacement of peridinin plastids[15]. Interestingly, molecular phylogenetic analyses indicate that the Apicomplexa are most closely related to the Dinoflagellata[16]. This suggests that the common ancestor for both these groups had three-membrane plastids and that apicoplasts result from plastid replacement[15]. A model of apicoplast evolution is provided by the unusual plastids of *Lepidodinium viride*, which, like those of apicomplexan plastids[3], are surrounded by four membranes and have a green algal origin[14,15]. It is possible that such an *L. viride*-like protist, possessing both three- and four-membrane plastids, was the ancestor of the Apicomplexa[15].

If apicoplasts have really evolved via the replacement of three-membrane plastids, their nuclear-encoded proteins should be imported co-translationally[5]. As the outermost membrane of the plastids is ribosome free, it appears that apicoplast proteins are first targeted into the ER, then to the Golgi apparatus and finally, with the use of pre-plastid vesicles, to the plastid[17]. The presence of signal peptides in the pre-sequences of these proteins[18] confirms the possibility of such a pathway in the apicomplexan cells.

The complex targeting mechanism of the apicoplast proteins might become a drug target in the future. A modification of SNARE proteins located on pre-plastid vesicles (v-SNAREs) and/or on the outermost apicoplast membrane (t-SNAREs) could effectively prevent the fusion of these vesicles with the apicoplast membrane[17]. Consequently, apicoplast proteins would be mistargeted to the endomembrane system and would never reach the plastid. The search for chemical factors that are able to modify SNARE proteins could be helped by an examination of endosymbiotic associations with pathogenic bacteria, such as *Mycobacterium*, *Chlamydia* and *Legionella*. Interestingly, all these microorganisms inhibit phagosome–lysosome fusion[19], one of the ways probably being by the secretion of SNARE-interacting proteins into the host cell[20].

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Response from McFadden and Waller

Bodył's hypothesis posits replacement of an incumbent plastid with a different plastid acquired through engulfment and retention of a photosynthetic eukaryote, a process known as secondary endosymbiosis. He suggests that both the nucleus-encoded genes and the targeting machinery of the incumbent plastid

could service the newly acquired plastid, thereby obviating the need for further gene transfer and reinventing complicated targeting of nucleus-encoded proteins into a plastid surrounded by multiple membranes. Such streamlined plastid evolution is argued to have enabled the multiple establishment of secondary endosymbionts in diverse lineages. Dinoflagellates are noted as a group in which 'replacement' appears to have resulted in endosymbionts of various colours and evolutionary affinities replacing the 'ancestral' peridinin plastid (bounded by three membranes).

Bodył's replacement hypothesis has several interesting implications. Plastids utilize ~1000 proteins, the majority of which are nucleus encoded[1]. Many of these proteins comprise multi-component systems. If a newly gained endosymbiont became import-competent to gene products destined for a different plastid, it would suddenly receive a flood of foreign proteins that would compete with indigenous proteins in the equivalent biochemical pathways. The replacement hypothesis requires that these 'outsider' proteins complement the indigenous subunits, which then become redundant. Certainly, many components of plastids are interchangeable[2,3], even from donors as distantly related as *Escherichia coli*[4], but the eventual loss of the endosymbiont nucleus would require the successful substitution of ~800–900 components. A turbulent period of cell evolution must be envisaged.

A further implication of the replacement hypothesis is that the new host nucleus must already contain all the genes not coded in the newly acquired plastid. However, allocation of genes between the nucleus and plastid genome is variable among different algae; one algal nucleus has a different cohort of plastid protein genes from another, and the more distant the algae, the more distinct the apportioning of genes between the two genomes[5]. Thus, even if imported foreign proteins could replace indigenous ones encoded by the endosymbiont nucleus, it seems unlikely that a complete complement would be represented. This conundrum could only be solved if the 'missing' gene(s) were relocated from the endosymbiont nucleus to the new host nucleus and their products targeted back to the plastid. This, however, is the evolutionary pathway that the replacement hypothesis seeks to avoid.

So how likely is it, as Bodył suggests, that plastid resampling has been a driver for the observed diversity of secondary endosymbionts? Not very. The replacement hypothesis is only more parsimonious if all the secondary endosymbiont-containing algae had a common plastid to replace, and that doesn't seem to be the case. Secondary endosymbiosis is widespread in eukaryotic diversity, with major algal groups such as kelps, diatoms, golden flagellates, haptophytes, euglenoids, cryptomonads, dinoflagellates and chlorarachniophytes all believed to have obtained their chloroplasts by secondary endosymbiosis[6]. However, in none of these lineages is there evidence for replacement. Indeed, for most of these lineages, the ancestral state is believed to be non-photosynthetic and totally lacking any plastid to replace[6].

More tantalizing, perhaps, is the case of the apicomplexan parasites, a group closely related to dinoflagellates. Apicomplexa have a four-membrane plastid, but Bodył suggests that this replaced a three-membrane plastid similar to the one found in dinoflagellates. The problem with this hypothesis is that in the earliest branching Apicomplexa there is still no evidence of any plastid, either three-membraned or four-membraned[7].

More critically for the replacement hypothesis, it is not clear that targeting of proteins across the three membranes of the incumbent dinoflagellate plastid is identical to the targeting across the four-membrane plastids of most secondary endosymbionts. How proteins cross the extra membrane is not yet clear, but our recent use of plastid targeting leaders to direct green fluorescent reporter protein (GFP) into the *Toxoplasma* plastid[8] provides a potential way to test this. A dinoflagellate-targeting peptide could be attached to GFP and introduced into *Toxoplasma*. If the GFP localizes in the *Toxoplasma* plastid, we can deduce that the three-membrane system is similar to the four-membrane system, and one theoretical obstacle for the replacement hypothesis would be removed.

In preference to replacement, we believe it is more likely that the genes of the endosymbiont nucleus gradually transferred to the nucleus of the new host, and we have identified two separate instances of such transfers in the chlorophyll-binding proteins of cryptomonad and chlorarachniophyte plastids[9]. As secondary endosymbiont targeting initially utilizes the standard eukaryotic secretory pathway (endosymbionts reside in endomembrane compartments), it is not necessary to reinvent the entire targeting mechanism each time[10]. Indeed, adapting the existing system seems to have allowed numerous establishments of secondary endosymbiosis.

Finally, Bodył proposes that the machinery for targeting proteins into plastids could be a useful drug target to combat diseases caused by plastid-containing parasites. However, we believe that other plastid pathways, such as DNA replication, transcription, translation, fatty acid biosynthesis, amino acid biosynthesis and haem synthesis (for which there are already known compounds that specifically inhibit function in plastids and sometimes parasites[7,8]), offer a richer pool of targets. We know of only one agent, azide, that inhibits plastid protein targeting *in vivo*[11], and its broad toxicity obviates utility as a therapeutic.

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