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Plastids and Protein Targeting[1]

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ABSTRACT. Plastids with two bounding membranes—as exemplified by red algae, green algae, plants, and glaucophytes—derive from primary endosymbiosis; a process involving engulfment and retention of a cyanobacterium by a phagotrophic eukaryote. Plastids with more than two bounding membranes (such as those of euglenoids, dinoflagellates, heterokonts, haptopytes, apicomplexa, cryptomonads, and chlorarachniophytes) probably arose by secondary endosymbiosis, in which a eukaryotic alga (itself the product of primary endosymbiosis) was engulfed and retained by a phagotroph. Secondary endosymbiosis transfers photosynthetic capacity into heterotrophic lineages, has apparently occurred numerous times, and has created several major eukaryotic lineages comprising upwards of 42,600 species. Plastids acquired by secondary endosymbiosis are sometimes referred to as "second-hand." Establishment of secondary endosymbioses has involved transfer of genes from the endosymbiont nucleus to the secondary host nucleus. Limited gene transfer could initially have served to stabilise the endosymbioses, but it is clear that the transfer process has been extensive, leading in many cases to the complete disappearance of the endosymbiont nucleus. One consequence of these gene transfers is that gene products required in the plastid must be targeted into the organelle across multiple membranes: at least three for stromal proteins in euglenoids and dinoflagellates, and across five membranes in the case of thylakoid lumen proteins in plastids with four bounding membranes. Evolution of such targeting mechanisms was obviously a key step in the successful establishment of each different secondary endosymbiosis. Analysis of targeted proteins in the various organisms now suggests that a similar system is used by each group. However, rather than interpreting this similarity as evidence of an homologous origin, I believe that targeting has evolved convergently by combining and recycling existing protein trafficking mechanisms already existing in the endosymbiont and host. Indeed, by analyzing the multiple motifs in targeting sequences of some genes it is possible to infer that they originated in the plastid genome, transferred from there into the primary host nucleus, and subsequently moved into the secondary host nucleus. Thus, each step of the targeting process in "second-hand" plastids recapitulates the gene's previous intracellular transfers.

Key Words. Endosymbiosis, intracellular gene transfer, plastids, protein targeting.

EUKARYOTIC cells carry out a variety of processes in their various compartments. Compartmentation, which is achieved largely through membrane boundaries, is essential for separating incompatible pathways, particularly opposing pathways such as glycolysis and the Calvin cycle which are essentially the reverse of each other. Successful establishment of the compartments requires targeting of protein components into the various compartments. We recognise two main categories of such targeting: export and import (Schatz and Dobberstein 1996).

Export describes the co-translational insertion of proteins across the endoplasmic reticulum membrane. This process is mediated by a *signal peptide* at the protein's N-terminus and results in the protein being deposited within the lumen of the ER from whence it is trafficked to other sectors of the endomembrane system or eventually secreted to the surface of the cell. This export system is clearly derived from the Sec-dependent secretory system of prokaryotes (Schatz and Dobberstein 1996), which supports the assertion that the whole endomembrane system, including the nuclear envelope, is derived from internalised and elaborated plasma membrane of a prokaryotic ancestor (Cavalier-Smith 1987).

Import mechanisms describe the transport of proteins into mitochondria and chloroplasts (also known as plastids) (Schatz and Dobberstein 1996). Mitochondria, and most plastids, have two bounding membranes, and transport occurs at specific sites where the two membranes are appressed. Complex machinery at the site serves as a gate and channel to admit only those proteins carrying an N-terminal extension referred to as a *transit peptide* (Heins, Collinson and Soll 1998). The origins of this import machinery are somewhat obscure. Cavalier-Smith (1982) has suggested that the plastid import mechanism derives from the pre-established mitochondrial import mechanism; although many components of the two systems have now been cloned, the only obvious homology is between the peripheral components such as chaperones. Indeed, the central component of the plastid system, Toc 75 which forms a gated channel across the outer membrane, has a homologue in cyanobacteria which is more consistent with a direct origin from the plastid endosymbiont (Heins et al. 1998).

Within mitochondria and plastids there is often a subsequent targeting system. Homologous to an *export* system, this targeting involves insertion of proteins into the plastid thylakoid lumen or between the inner and outer membranes of mitochondria. This intra-organelle targeting is a residue of the bacterial export system and involves a bacterial *signal peptide* (Hartl and Neupert 1990; Settles et al. 1997; Yaun et al. 1994). For nuclear-encoded proteins, this signal lies downstream of the transit peptide and swings into action only after removal of the transit peptide in the organelle matrix (Schatz and Dobberstein 1996).

A less well understood targeting system occurs in many algae and protists. These organisms—which include diatoms, brown seaweeds, euglenoids, dinoflagellates, cryptomonads, chlorarachniophytes, apicomplexa, and haptopytes—have chloroplasts with three or four bounding membranes (Palmer and Delwiche 1996). These multi-membraned chloroplasts also import proteins, but they do it in a most unusual way—by combining both the *export* and *import* systems. This article will outline what we know about this double-barrelled transport system, but in order to understand the process fully, we must first explore two other phenomena: endosymbiosis and intracellular gene transfer.

Gobbling cells and jumping genes. Mitochondria and chloroplasts clearly arose by endosymbiosis where free-living bacteria took up residence in nucleated cells. Mitochondria trace their ancestry to α-purple bacteria (organisms like *Agrobacterium, Rickettsia, Rhizobium*) and chloroplasts derive from cyanobacteria (Gray 1992). During their tenure in the host, both mitochondria and plastids began to lose autonomy. A key factor in this diminishing autonomy has been the loss of much of their genetic material (Palmer 1991). As endosymbionts, sheltered and provided for within a eukaryote host, the bacteria probably had no further use for many of their original functions. These presumably were lost. However, it has also emerged that many of the genes for proteins essential to the organelle have been

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transferred to the nucleus of the host. Why did this happen? While a definite answer can't yet be given, a few hypotheses are on offer. At the outset, an endosymbiosis involves two independent entities. If the partnership is beneficial to both members, we can postulate selection for any event that prevents the partners dispersing and returning to individual existences. Loss of autonomy, such as would occur by transferring a gene to the host, is just such an event. In other words, selection would favor a partnership in which the host nucleus confiscates endosymbiont genes (Howe 1996). The resultant knobbling of the bacterial guest cell would ensure that it remained within the host thereby locking the partners together in beneficial symbiosis. Indeed, the working definition of an endosymbiont requires that some of its genetic material be transferred to the host genome (Cavalier-Smith and Lee 1985).

While this "lock in" argument seems highly plausible, it can only be used to rationalise the transfer of a limited amount of genetic material: a single endosymbiont gene would be sufficient in most instances. But the transfer has been substantial and continues inexorably. Chloroplasts are guesstimated to contain about 1,000 proteins. About 900 of these proteins would be nuclear-encoded since the chloroplast encodes only about 100. Similarly, mitochondria contain 500–600 proteins and the mitochondrion encodes only a handful of proteins so most must be nuclear-encoded. In yeast, this means that 5%–10% of the organism's 6,000 genes are for proteins targeted to the mitochondrion (*http://www.proteome.com/search1.html*). Almost all of these are genes that moved from the endosymbiont to nucleus. Why so many? As endosymbionts, mitochondria and chloroplasts embarked on a very narrow and tortuous genetic path. Locked up inside a host cell they had little opportunity for recombination (but, see Köhler et al. 1997) and became part of a relatively small population. Worse still, they were subject to uniparental inheritance and increased mutation rates due to the high levels of active oxygen species and free radicals produced by photosynthesis (Allen and Raven 1996) and respiration respectively (de Gray 1997; Lee and Wei 1997). This heightened mutation load and limited capacity for correction in haploid (albeit multicopy) genomes led to severe pressure on the coding capacity, and organelle genes are now recognized to be among the most divergent. But, those organelle genes that decamped to the host nucleus found themselves in an entirely different milieu. Genes in the nucleus enjoy frequent recombinations, low mutation rates, and diploidy to compensate for deleterious recessives. Clearly, there would be genetic advantages to relocating genes into the host genome, but while coding fidelity was probably a key factor in much intracellular gene movement, the reverse was probably also true. Copying genes into the nucleus created redundancy. If an organelle gene was still in place, then the nuclear copies were excess to requirements. These excess genes, released from functional selection, were potentially free to explore genetic space. Nuclear copies of organelle genes were liberated and thus, able to pass through multiple deleterious mutations that might eventually lead to a more fit gene unable to be reached by the incumbent organelle copy. If the product of this new gene in the nucleus could but find the keys to the door and return to the organelle, it could eventually supplant its organelle progenitor (Weeden 1981). But what were the mechanics of this process? How did the DNA move? How did the gene 'find' a fitter alternative before decaying into useless garbage DNA? How did the product obtain the keys to door and get to the organelle?

Drip, drip, drip. Transfer of the gene seems relatively straightforward. Plastids and mitochondria are typically present in multiple copies. If one organelle breaks and the DNA is released into the cytoplasm, then it could easily integrate into nuclear chromosomes. Transformation technology tells us that any DNA that gets into a cell—whether it is shot in, injected in, or zapped in by electricity—can wind up in the nucleus. The organelle DNA only has to escape from its compartment, which seems relatively facile. Indeed, some clever experiments have shown that organelle DNA finds its way into the nucleus at astonishing rates (Blanchard and Schmidt 1996a; Brennicke et al. 1993; Hu and Thilly 1994; Martin et al. 1998; Palmer 1991). For instance, Thorsness and Fox (1996) placed a selectable marker into the mitochondrial genome of yeast, but the marker was dysfunctional in mitochondria as its expression was driven by a nuclear promoter—only copies that relocated to the nucleus could produce a product. Challenge with the selective agent yielded one resistant individual (a mitochondrion to nucleus gene transfer) every 10^5 divisions, and this is the rate for a single locus not the entire mitochondrial genome (Thorsness and Fox 1996). Similar experiments are yet to be done for chloroplasts, but examination of the genomic sequence databases revealed that between 3%–7% of the entries for nuclear DNA also contained fragments of chloroplast or mitochondrial DNA apparently transferred sufficiently recently to be still recognisable as 'contaminants' of bona fide nuclear DNA (Blanchard and Schmidt 1996a). These fragments of the organelle genomes may have undergone direct transfer as DNA or via an RNA intermediate (Blanchard and Schmidt 1996b; Brennicke et al. 1993). A recent hypothesis (Yoshida et al. 1995) even suggests that DNA transfer is concerted and that the organelle and nucleus have a form of trans-kingdom sex in which the organelle unloads its genes into the host genome. Thus, frequent and relentless (think in terms of hundreds of millions of years here) gene transfer provided a continual trickle of organelle DNA into the nucleus (Martin et al. 1998; Thorsness and Weber 1996). Most of this DNA probably decays into unrecognizable junk, but the occasional pieces probably stumble into useful genetic space, perhaps even hitting on superior permutations.

Transit peptides: keys to the organelle doors. Studies of the *import* mechanisms have shown that the N-terminal transit peptide is sufficient to target virtually any protein into an organelle (Heins et al. 1998; Schatz and Dobberstein 1996) so the nuclear encoded products need only acquire a transit peptide to reach the organelle, assuming they are able to be expressed and translated in their novel milieu. How likely is it that a gene landing somewhere in the nucleus will end up with a transit peptide? Surprisingly likely. Hundreds of transit peptides have now been characterised, but they defy pigeonholing (Schneider et al.1998; Von Heijne 1991). No primary consensus exists, and, although mitochondrial transit peptides have a structural basis with an amphipathic helix, chloroplast transit peptides have no identifiable structural similarity and apparently rely on a high frequency of a few amino acids (serine, threonine, glycine, and proline) and a net positive charge for function (Von Heijne 1991; Von Heijne et al. 1991). Transit peptides are also very disparate in size ranging from 25–120 amino acids for chloroplasts (Von Heijne et al. 1991). Thus, if the constraints for targeting are so loose, it is conceivable that many pieces of sequence might suffice to target proteins to organelles. Indeed, a trial using random sequence in place of transit peptides demonstrated that a whopping 10% of random sequences were competent to act as a transit peptide (Allison and Schatz 1986). While this statistic suggests that organelles must probably cope with all sorts of incoming material falsely directed their way by bogus transit peptides, it also means that transferred pieces of organelle DNA have a surprisingly high probability of holding *the keys to the door* if they land, or end up, in frame behind a reasonably appropriate piece of sequence in the nucleus. For example, a single transversion has been shown to create a new

start codon 18 amino acids upstream of a gene in yeast, and the extra N-terminal sequence acts as a transit peptide targeting the protein to the mitochondrion (Harrington et al. 1994). There is also evidence that successful transit peptides are recycled (Kadowaki et al. 1996), sometimes even by the process of exon shuffling (Long et al. 1996; Wegener and Schmitz 1993; Wischmann and Schuster 1995), and in one instance a transit peptide is appended to the N-terminus of a chloroplast protein by differential splicing (Thorbjornsen et al. 1996).

Going, going, gone. Given that gene transfer and return product targeting seem so facile, it is reasonable to ask why any genes remain in chloroplasts and mitochondria at all. The nucleus apparently represents safe haven to these genes, and maintenance of organelle transcription and translation systems seems a heavy burden for the production of a minority of constituents, so selection ought to favor complete transfer and the ultimate disappearance of organelle genomes. Borst (1977) suggested that perhaps some proteins are simply too hydrophobic to be trafficked and must be synthethised at the site of incorporation. In this respect it is probably salient that two extremely hydrophobic mitochondrial proteins, Cox1 and Cytb, are encoded by all known mitochondria (Palmer 1997a; 1997b); their persistence in mitochondria lends much credence to the too-hydrophobic-to-transfer hypothesis (Claros et al. 1995; Von Heijne 1986). On the other hand, ultra-hydrophobic proteins like the large subunit of Rubisco were also thought to be trapped forever in the compartment of use. But some straightforward genetic engineering, in which *rbcL* was deleted from the chloroplast and successfully relocated to the nucleus and the product targeted to the chloroplast (Kanevski and Maliga 1994), showed that the hydrophobicity argument doesn't always hold. Perhaps some genes just never got around to being relocated. Mapping of gene transfers during plastid evolution suggests a high level of *ad hocery* (Martin et al. 1998).

Another line of evidence supporting hydrophobicity anchoring comes from anaerobic protists such as the parasite *Trichomonas*. These flagellates contain a highly modified mitochondrion that no longer contains an electron transport chain (Müller 1997). Instead, the organelle converts pyruvate to acetate and produces H_2 as a byproduct. Known as hydrogenosomes, these organelles are clearly derived from mitochondria, but they have no DNA. A simple rationalisation of this is that their anaerobic lifestyle rendered the ultra-hydrophobic electron transport chain proteins (Cox1 and Cytb) useless allowing the genome to disappear (Palmer 1997a). A similar explanation can be made for yeast rho and petite mutants, which also lose the mitochondrial genome when existing anaerobically.

Double engulfing: genes on the move again. The primary endosymbiotic establishment of plastids in the eukaryotic lineage leading to plants left other eukaryotes (fungi, animals, and many protozoa) without photosynthesis. This missed opportunity means that animals and fungi must scavenge the environment for food because they can't make it. But select protozoan lineages managed to acquire a plastid second-hand by engulfing a plastid-containing cell and retaining it (Fig. 1). In some organisms (cryptomonads and chlorarachniophytes) we can still see traces of this process in the persistent vestigial nucleus and cytoplasm of the endosymbiont (Gilson et al. 1997), but in most cases the only evidence of the secondary endosymbioses are the multiple membranes now surrounding the plastid. (Fig. 1). In cases where four membranes surround (the second-hand) plastid, the inner pair of membranes corresponds to the original two plastid membranes, the third membrane (counting from the inside) corresponds to the plasma membrane of the endosymbiont, and the outermost membrane derives from the phagosome (Fig. 1). This means that topologically the endosymbiont

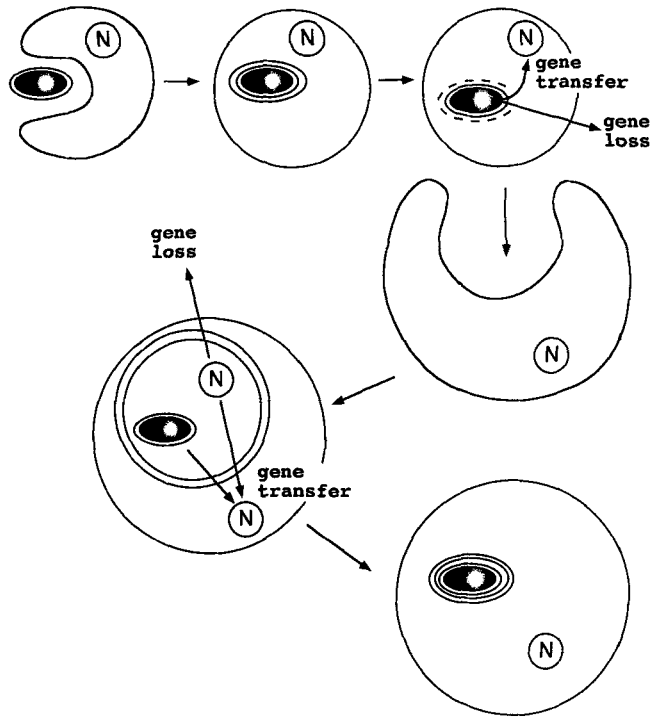

Fig. 1. Sequential endosymbiotic events (primary and secondary) producing plastids. A primary plastid arises from engulfment and retention of a photosynthetic prokaryote. The phagosomal membrane ruptures releasing the endosymbiont, with its two membranes, into the cytoplasm. Products of genes transferred from the prokaryote to the eukaryote host nucleus are targeted back to the endosymbiont by an *import* mechanism using a transit peptide. Plastids acquired by secondary endosymbiosis have four bounding membranes. Genes transfer to the secondary host nucleus from the endosymbiont nucleus, which eventually disappears leaving only the membranes. Targeting of the proteins from the secondary host to the endosymbiont initially utilises the *export* mechanism because the outermost membrane of the plastid derives from the phagosome.

is located "outside" the host since it is within the lumen of the endomembrane system.

At the time of engulfment, we predict that the endosymbiont nucleus harboured numerous genes for plastid proteins, so retention of the endosymbiont nucleus was probably essential for maintenance of the plastid initially (Gilson et al. 1997) (Fig. 1). These proteins would have carried N-terminal leaders (transit peptides) for plastid targeting/import. However, it seems unlikely that the endosymbiont nucleus could continue to reproduce sexually inside the host; the complications of syngamy within the confines of a host cell are immense. The endosymbiont nucleus was thus in a similar genetic dilemma to the chloroplast and mitochondrial genomes; limited opportunity for recombination and a high mutational load due to population bottleneck. Secondary endosymbiosis thus leads to a second round of intracellular gene transfers, this time from the endosymbiont nucleus to the host nucleus. Given that an endosymbiont nucleus persists in only two of the posited six secondary endosymbioses, this transfer was apparently highly successful (Palmer 1997a).

What obstacles confront the vagrant protein from a second-hand plastid? As outlined in Fig. 1, four membranes separate the host cytoplasm from the plastid matrix, and the outermost apparently derives from the endomembrane system. The simplest solution for crossing this membrane was apparently to

Table 1. List of examples of cloned nuclear genes encoding plastid proteins in algae with multiple membrane chloroplasts. Most, but apparently not all, of the predicted proteins carry a complex (at least bipartite) N-terminal extension that probably effects transport into the plastid across the multiple bounding membranes.

Group	Genus	Gene	Protein	Reference
Euglenozoa	*Euglena*	*gapA*	Chloroplast glyceraldehyde-3-phosphate dehydrogenase	(Hene et al. 1998)
	Euglena	CA*B*	Chlorophyll a/b binding protein	(Kishore et al. 1993; Sulli & Schwartzbach 1995; 1996)
	Euglena	*rbc*S	Rubisco small subunit	(Chan et al. 1990)
	Euglena	PBGD	Porphyrinobiligen diaminase	(Sharif et al. 1989; Shashidhara & Smith 1991; Shashidhara et al. 1992)
Bacillariophyceae	*Odontella*	*atpC*	γ subunit of plastid FoFi ATPase	(Pancic & Strotmann 1993)
	Odontella	FCP	Fucoxanthin chlorophyll c binding protein	(Kroth-Pancic 1995)
	Phaeodactylum	FCP	Fucoxanthin chlorophyll c binding protein	(Apt et al. 1994; Bhaya & Grossman 1991; Grossman et al. 1995; Grossman et al. 1990)
	Odontella	PRK	Phosphoribulokinase	(Lang et al. 1998)
Phaeophyceae	*Macrocysitis*	FCP	Fucoxanthin chlorophyll c binding protein	(Apt et al. 1995)
	Laminaria	FCP	Fucoxanthin chlorophyll c binding protein	(Caron et al. 1996)
Raphidophyceae	*Heterosigma*	FCP	Fucoxanthin chlorophyll c binding protein	(Durnford et al. 1996)
Chrysophyceae	*Giraudyopsis*	FCP	Fucoxanthin chlorophyll c binding protein	(Passaquet & Lichtl 1995)
Haptophyceae	*Isochrysis*		Fucoxanthin chlorophyll c binding protein	(Laroche et al. 1994)
Dinoflagellata	*Amphidinium*	PCP	Peridinin chlorophyll c binding protein	(Sharples et al. 1996)
	Gonyaulax	*rbcL*	Type II ribulose bisophosphate carboxylase	(Morse et al. 1995)
	Gonyaulax	*gapC*	Chloroplast glyceraldehyde-3-phosphate dehydrogenase	(Fagan et al. 1998)
	Heterocapsa	PCP	Peridinin chlorophyll c binding protein	(Sharples et al. 1996)
	Symbiodinium	PCP	Perdinin chlorophyll c binding protein	(Norris & Miller 1994)
	Symbiodinium	*rbcL*	Type II ribulose bisphosphate carboxylase	(Whitney et al. 1995)
Cryptophyta	*Pyrenomonas*	*gapC*	Chloroplast glyceraldehyde-3-phosphate dehydrogenase	(Liaud et al. 1996)
	Chroomonas	*cpeA*	Alpha phycoerythin	(Jenkins et al. 1990; McFadden & Gilson 1997)
	Guillardia	CAC	Chlorophyll a/c binding protein	(Deane & McFadden 1999, unpubl.)
Chlorarachmiophyta	*Chlorarachnion*	CAB	Chlorophyll a/b binding protein	(Deane & McFadden 1999)
	Chlorarachnion	*gapX*	Chloroplast glyceraldehyde-3-phosphate dehydrogenase	(Petersen & Cerff 1998)

utilise the pre-existing *export* process (Gibbs 1981). Genes for host nucleus-encoded plastid proteins have now been cloned from each of the various groups of organisms thought to have second-hand plastids (Table 1). In almost every case the proteins carry an N-terminal extension that conforms to an *export* or *signal* peptide. Interestingly, there is an additional motif downstream of the signal peptide and this motif generally bears the hallmarks of a transit peptide or import signal (Reith 1996). These sequences suggested that the first component of targeting is an *export,* from the host to the lumen of the endomembrane system, and the second component is an *import,* from the vestigial cytoplasm of the endosymbiont into the plastid (Fig. 2). The simplest interpretation is that the translocated genes acquired a signal peptide at their N-terminus when they relocated to the host nucleus. Signal peptides are a little more exacting than transit peptides in primary sequence but they are also relatively loose in terms of consensus and size (Nielson et al. 1997). In at least a few cases, the signal peptide of plastid proteins comprises an exon (Caron et al. 1996; Waller et al. 1998) perhaps suggesting acquisition by exon shuffling.

Getting a foot in the door. Experimental evidence for this double-barrelled trafficking is limited. In euglenoids it has been possible to observe plastid proteins in the Golgi apparatus by immunogold electron microscopy (Osafune et al. 1991) and pulse chase cell fractionation (Sulli and Schwartzbach 1996). Similarly, utilisation of the export system seems likely in diatoms since a signal peptide domain at the N-terminus of diatom plastid proteins is effective in heterologous assay systems (Apt et al. 1994; Bhaya and Grossman 1991; Grossman et al. 1995; Grossman et al. 1990).

While this seems to be a neat way of targeting products of relocated genes to second-hand plastids, there is of course, one step missing. After secretion into the lumen of the endomembrane system, the protein is essentially outside both the host and the endosymbiont (Fig. 2). How does the protein cross the relict plasma membrane of the endosymbiont? Nothing is known about this step and it is particularly intriguing since it requires a transport step from outside to inside (a type of import?), which occurs only sparingly as far as we know. One possibility is that the endosymbiont plasma membrane is porous and acts as a relatively open sieve (Fig. 2), but this hypothesis then begs the question of why this membrane should persist if it has no role as a barrier. It is also difficult to rationalise in cryptomonads and chlorarachniophytes since the endosymbiont cytoplasm would leak out of a porous endosymbiont plasma membrane. But the sieve hypothesis is perhaps germane to the number of membranes surrounding euglenoid and dinoflagellate plastids. While it is widely held that these plastids have a sec-

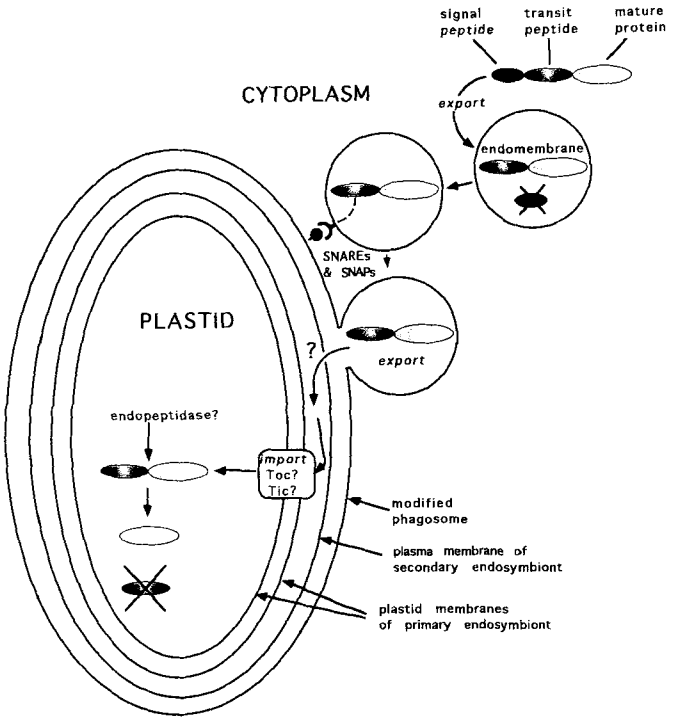

Fig. 2. A model for targeting host-encoded proteins into a second-hand plastid like those of apicomplexa or chlorarachniophytes. The protein has a bi-partite leader comprising a signal peptide and a transit peptide. The signal peptide effects co-translational insertion of the precursor into the lumen of the rough ER (endomembrane system). The signal peptide is probably removed at this stage. A tag, which resides in the transit peptide domain, results in the vesicle cargo being diverted out of the secretory pathway and toward the plastid. The vesicle is believed to fuse with the outermost membrane of the plastid, perhaps using special SNAREs and SNAPs, delivering the cargo into the space between the outer pair of membranes. The protein somehow crosses the third (counting from the inside) membrane, which perhaps has large pores. The transit peptide then interacts with the second membrane and possible receptors localised in this membrane, and engages an import apparatus homologous to the Toc and Tic system of plant chloroplasts which translocates the protein across the inner pair of membranes (Heins et al. 1998). Within the plastid an endopeptidase cleaves the transit peptide which is degraded.

ondary origin (Delwiche and Palmer 1997; Gibbs 1978; McFadden and Gilson 1995; Palmer and Delwiche 1996), Cavalier-Smith (1982) has pointed out that three membranes could arise from a primary endosymbiosis (look at the earliest stage in Fig. 1). A primary origin for these plastids is not inconsistent with targeting initially using an export system since the outermost membrane would be part of the host's endomembrane (see Fig. 1). However, if euglenoid and dinoflagellate plastids have a primary origin, we must infer independent transfer of all plastid genes to their host nuclei. The frequency of gene transfer (Martin et al. 1998) means that this is not impossible, but the targeting complexities lead me to believe that it didn't happen this way. In secondary endosymbiosis the complex targeting arose in a two-step process, but for primary plastids with three membranes the two steps (export and import) would have to arise simultaneously. Even after both sets of machinery for targeting to secondary plastids were established, each transferred gene would still need to acquire two separate motifs (signal peptide and transit peptide) to achieve return passage to the plastid. This, along with other data (Delwiche and Palmer 1997; Hallick et al. 1993; Palmer and Delwiche 1998), suggests to me that euglenoids and dinoflagellates have secondary plastids. Schnepf (1993) pointed out that the unusual myzocytoic feeding process in dinoflagellates could explain the three membranes around their plastids, but this feeding mechanism is unknown in euglenozoans, which use classic engulfment via a cytostome. An intriguing possibility is that euglenoids had a porous, sieve-like endosymbiont plasma membrane to facilitate transport as hypothesised above, and that they dispensed with this redundant barrier to reduce the number of surrounding membranes from four to three.

An experimental system for double-barrelled targeting. While various algal gene sequences provided a model for targeting to second-hand plastids (Bodyl 1997), there was no useful system to explore the process experimentally—none of these organisms is able to be transformed and most are not genetically tractable. Recently though, an excellent model for plastid targeting has emerged from an unlikely quarter. When a vestigial plastid was discovered in human parasites like malaria and *Toxoplasma* (Köhler et al. 1997; McFadden et al. 1996; Wilson et al. 1996), it became appropriate to ask if any nuclear genes encode proteins that are trafficked into this plastid. The plastid in these parasites has four bounding membranes and is believed to have a secondary origin (Köhler et al. 1997). My laboratory has examined a number of nuclear genes from *Plasmodium* and *Toxoplasma,* and demonstrated that the products are targeted into the plastid (Waller et al. 1998). In line with the model, these proteins carry bi-partite N-terminal leaders comprising a signal peptide and a transit peptide (Waller et al. 1998). Western blots (Waller et al. 1998) demonstrate that the leader is removed to produce a mature protein of the expected size (Fig. 2).

Unlike most algae, *Toxoplasma* is amenable to experimental manipulation and transformation, and reporter protein studies can be undertaken (Striepen et al. 1998). This enabled us to collaborate with the group of David Roos and test if the bipartite leader was sufficient to target the green fluorescent reporter protein (GFP) into a plastid. Successful targeting was achieved using the leader (Waller et al. 1998) and a number of different constructs have allowed us to dissect this leader experimentally. Deletion of the transit peptide downstream of the signal peptide resulted in secretion of GFP, thereby demonstrating that the signal peptide indeed routes the proteins through the endomembrane system and that information in the transit peptide is essential to divert the cargo out of the secretory pathway and toward the endosymbiont (Fig. 2). Is the signal peptide in plastid targeting leaders different to regular signal peptides for general export? It seems not. When a transit peptide was inserted between a signal peptide for a secreted protein and GFP (i.e. standard signal peptide/transit peptide/reporter protein) the reporter was trafficked to the plastid confirming that transport is via the endomembrane system and that the transit peptide effects diversion out of the secretory pathway. Deletion experiments also demonstrate that both components (*export* and *import*) are necessary for plastid targeting. If the signal peptide is absent, the transit peptide alone fails to effect targeting, and the GFP accumulates in the cytoplasm. These experiments demonstrate that the bipartite leader is sufficient and necessary for targeting proteins to the secondary endosymbiont in *Toxoplasma.* The system provides a convenient means to determine what motifs are essential for plastid targeting. It is not yet clear whether the proteins target via the Golgi apparatus; this awaits electron microscopic localisation of intermediates.

Summary. Relocation of endosymbiont genes into the host genome after engulfment has necessitated acquisition of targeting signals to return the gene product to its compartment of origin; an *import* signal in the case of transfer between prokaryotic endosymbionts and primary host nuclei, plus an ad-

ditional *export* signal in cases where genes have subsequently been transferred from an endosymbiont nucleus to a host nucleus in secondary endosymbioses. In extreme cases (McFadden and Gilson 1997), proteins targeted from the host into the thylakoid lumen of second-hand plastids combine three targeting systems (*export, import,* and prokaryotic *export*) to cross five membranes! These tandemly arrayed targeting motifs serve as labels telling us about previous homes of the genes, and, in a sense, the separate targeting steps recapitulate the gene transfer events.

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ADDENDUM

Subsequent to the preparation of this review several topical papers (Akhmanova et al. 1998; Arjan et al. 1999; Bodyl 1999;

Doolittle 1998; Lang et al. 1998; Lynch and Blanchard 1998; Martin and Herrmann 1998; Martin and Müller 1998; Sulli et al. 1999) have been published.

Akhmanova, A., Voncken, F., van Alen, T., van Hoek, A., Boxma, B., Vogels, G., Veenhuis, M. & Hackstein, J. H. P. 1998. A hydrogenosome with a genome. *Nature,* **396**:527–528.

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