Der1-mediated Preprotein Import into the Periplastid Compartment of Chromalveolates?

Maik S. Sommer,* *Sven B. Gould*,¹* *Petra Lehmann*,* *Ansgar Gruber*,‡ *Jude M. Przyborski*,§ *and Uwe-G. Maier**

*Laboratory for Cell Biology, Philipps-University of Marburg, Marburg, Germany; ‡Department of Biology, University of Konstanz, Konstanz, Germany; and \$Laboratory for Parasitology, Philipps-University of Marburg, Marburg, Germany

Phototrophic chromalveolates possess plastids surrounded by either 3 or 4 membranes, revealing their secondary endosymbiotic origin from an engulfed eukaryotic alga. In cryptophytes, a member of the chromalveolates, the organelle is embedded within a designated region of the host's rough endoplasmic reticulum (RER). Its eukaryotic compartments other than the plastid were reduced to the mere remains of its former cytosol, the periplastid compartment (PPC, PP space), and its nucleus, the nucleomorph, separated from the RER by its former plasma membrane, the periplast membrane (PPM). In the nucleomorph genome of the cryptophyte Guillardia theta, we identified several genes sharing homology with components of the ER-associated degradation (ERAD) machinery of yeast and higher eukaryotes, namely ORF201 and ORF477, homologs of membrane-bound proteins, Der1p (Degradation in the ER protein 1) and the RING-finger ubiquitin ligase Hrd1, and a truncated version of Udf1, a cofactor of Cdc48, a lumenal ATPase. Exemplarily, studies on the Der1homolog ORF201 showed that this protein partially rescued a yeast deletion mutant, indicating the existence of a functional PPC-specific ERAD-like system in cryptophytes. With the noninvestigated exception of haptophytes a phylogenetically and mechanistically related system is apparently present in all chromalveolates with 4 membrane-bound plastids because amongst others, PPC-specific Derlins (Der1-like proteins), CDC48 and its cofactor Ufd1 were identified in the nuclear genomes of diatoms and apicomplexa. These proteins are equipped with the required topogenic signals to direct them into the periplastid compartment of their secondary symbionts. Based on our findings, we suggest that all chromalveolates with 4 membrane-bound plastids express an ERAD-derived machinery in the PPM of their secondary plastid, coexisting physically and systematically adjacent to the host's own ERAD system.

We propose herewith that this system was functionally adapted to mediate transport of nucleus-encoded PPC/plastid preproteins from the RER into the periplastid space.

Introduction

Two membrane-bound (primary) plastids evolved in primary endosymbiosis by enslaving a phototrophic cyanobacterium within a heterotrophic eukaryote about 850 MYA (Cavalier-Smith 2002, 2006; Hjorth et al. 2005). Plastids of chromalveolates (e.g., cryptophytes, heterokontophytes, haptophytes, and apicomplexa), in contrast, are surrounded by more than 2 envelope membranes, therewith revealing their secondary endosymbiotic ancestry. Having emerged from the phagotrophic engulfment and conversion of a primarily evolved red alga (outlined in Hjorth et al. 2005) they are, in most cases, surrounded by 2 additional membranes. In cryptophytes and heterokontophytes, the outermost membrane is continuous with the rough endoplasmic reticulum (RER) of the host (Gibbs 1979, 1981) and is believed to have originated by fusion of the food vacuole with the ER membrane (Cavalier-Smith 2003). The subsequent membrane, the periplastid membrane (PPM) is the former plasma membrane of the symbiont. Subjugation of these organelles ended in massive genome rearrangement, many genes having either been lost or transferred to the host nucleus. Their plastid genomes are thus more restricted in their coding capacity than those of modern red algae, and the nuclear genome of the secondary symbiont was in most cases completely lost (Stoebe and Maier 2002).

¹ Present address: School of Botany, University of Melbourne, Victoria, Australia.

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E-mail: maier@staff.uni-marburg.de.

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Among the plastid-bearing chromalveolates, cryptophytes are exceptional in that they exhibit more relics of their evolutionary past than all others: Remnants of the cytoplasm, the periplastid compartment (PPC) and the nucleus, the nucleomorph (Nm), of the secondary symbiont still exist between the outer and inner membrane pairs (Stoebe and Maier 2002). Due to the coexistence of the 2 cytoplasms (the host's cytoplasm and the PPC) and the restricted coding capacity of the Nm (only about 450 ORFs encoded, Douglas et al. 2001), protein trafficking is even more sophisticated in cryptophytes than in other chromalveolates because the host must complement not only the plastid but also the PPC with factors. Thus, 2 groups of nucleus-encoded proteins destined for the symbionts' compartments exist. We recently demonstrated that both PPC and plastid proteins, possess a bipartite topogenic signal (BTS), comprised of an N-terminal signal peptide and a plastid transit peptide, the sequence of which determines whether 2 membranes (into the PPC) or 4 membranes (into the plastid stroma) are crossed: An aromatic amino acid (aa) at position +1 of the transit peptide (TP) (predominantly a phenylalanine) facilitates the passage across the plastid envelope, whereas proteins without this conserved aa are retained and processed in the PPC (Gould, Sommer, Hadfi, et al. 2006; Gould, Sommer, Kroth, et al. 2006).

Beyond this, mechanistic details of preprotein import into secondary symbionts of chromalveolates remain poorly understood. The outermost of the 4 membranes surrounding the secondary symbiont of cryptophytes and heterokontophytes is continuous with the host RER, it is endowed with 80S ribosomes, as shown by the means of electron microscopy. Evidence suggests that proteins cross the outer membrane cotranslationally (Wastl and Maier 2000; Kilian and Kroth 2005; Gould, Sommer, Hadfi,

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The transport mechanism acting at the PPM is still enigmatic. To date, 2 translocon-based import models (Cavalier-Smith 2003; Bodyl 2004) and a vesicular shuttle system (Kilian and Kroth 2005) have been proposed. Once having entered the periplastid space, the TP of the BTS is responsible for sorting plastid and PPC proteins from the secretory pathway (Gould, Sommer, Kroth, et al. 2006).

In order to investigate the import mechanisms acting on the PPM of the secondary symbiont of the cryptophyte Guillardia theta, we first screened its Nm genome for genes encoding membrane proteins putatively involved in any kind of protein transport. Surprisingly, we identified 2 proteins being homolog to components of the endoplasmic reticulum-associated protein degradation (ERAD) machinery, encoded by other eukaryotes (Romisch 2005). Complementation assays performed in yeast indicate the existence of a second symbiont-specific ERAD-like system in cryptophytes. Interestingly, homologs of the Nm-encoded proteins identified in G. theta are also present among other chromalveolates, such as diatoms and apicomplexans, being nuclear encoded as preproteins with the required topogenic signals for their import into the PPC. Our data imply the existence of a conserved ERAD-like system in the PPC of 4 membrane-bound plastids of chromalveolates, which might be involved in preprotein translocation into this compartment.

Material and Methods

Data Mining and In Silico Analyses

Data mining for ERAD components was based on the Saccharomyces cerevisiae model. Protein sequences were obtained from the Saccharomyces Genome Database (http://www.yeastgenome.org/). All described Nmencoded ORFs of G. theta CCMP327 can be obtained from the National Center for Biotechnology Information (NCBI) Database (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). To identify the homologous genes in the genome of *Phaeo*dactylum tricornutum CCAP1055/1, publicly available single-read raw sequences of the Phaeodactylum tricornutum sequencing project were downloaded from the NCBI Trace Archive (ftp://ftp.ncbi.nih.gov/pub/TraceDB/phaeodactylum tricornutum/). Local Blast (Altschul et al. 1997) searches were done with heterologous queries of interest against the *Phaeodactylum* raw sequence database using the program BioEdit (Hall 1999). Starts and ends of the hit sequences were used as queries in iterative Blast searches against the Phaeodactylum raw sequence database to extract a subset of sequences from this database by the DOS-software geneworm (courtesy of Oliver Kilian). These single-read sequences were assembled into contigs by use of DNASTAR's SeqMan II software. The resulting contigs were checked for open reading frames (ORFs) with homology to the initial heterologous query of interest. All gene models are available at PhaeodactylumDB v2.0 (http://genome.jgi-psf.org/Phatr2/Phatr2.home.html). Screening of the genomes of *Plasmodium falciparum* 3D7 was performed using PlasmoDB v5.1 (http://www.plasmodb.org/plasmo/home.jsp) and standard search Blast tools (NCBI). All genes of interest found were analyzed using the Sequencher software v4.5 from Genecodes (Michigan, United States). Amino acid alignments were carried out using ClustalX v1.8 (NCBI) and BTS-prediction with the services offered by the CBS-prediction server (SignalP v3.0, TargetP v1.1; http://www.cbs.dtu.dk/services/) or for *P. falciparum* with PlasmoAP (http://v4-4.plasmodb.org/restricted/PlasmoAPcgi.shtml). Transmembrane domains (TMDs) were predicted with TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). All genes/gene models can be obtained upon request.

Pulse-Chase Experiments

Yeast strains used in this study were S. cerevisiae W303-1C (Mat α ade2-1 ura3-1 his2-11, 15 leu 2-3, 112 trp1-1 can1-100 prc1-1), W303-CD (W303-1C der1:: URA3) (Knop et al. 1996), and YRH110 (W303-1C der1:: URA3 ire::kanR) (Hitt and Wolf 2004). The plasmid used for overexpressing the yeast *der1* and *G*. *theta orf201* was pRS424TDH (2µ Trp1 ampR P_{TDH3} T_{CYC1}) (Funk et al. 2002). All genes were amplified in standard polymerase chain reaction (PCR) from genomic or cDNA with specific primers with restriction-site adaptors for EcoRI/XhoI and subsequently cloned into pRS424TDH using standard cloning techniques. Yeast was transformed with the LiAc method (Gietz et al. 1992). Pulse-chase experiments were essentially carried out as described in (Finger et al. 1993). Cells were grown to a midlogarithmic phase in Shine-Dalgarno (SD) media (supplements as required). 3.3 OD₆₀₀ unit cells were harvested for each time point measured and resuspended in SD medium without amino acids at a cell density of 10 OD_{600} unit/ml. After 30 min of starvation, pulse was given by adding (35S) methionine/ cysteine to a final concentration of 165 µCi/ml (Redivue-Promix, GE Healthcare, Munich, Germany) for 15 min. Cells were chased with an equal volume of SD medium with 75 µM methionine/cysteine. Equal volumes of cell suspension were taken at zero minutes and at each time interval. The reaction was stopped by adding 10 mM sodium azide. Cell rupture, protein solubilization, and immunoprecipitation were executed in accordance with Biederer et al. (1996). Mutated version of carboxypeptidase Y (CPY*) was precipitated by adding 0.5 µg mouse monoclonal anti-CPY antibody (Molecular Probes, Invitrogen, Karlsruhe, Germany) and 50 µl 3.5% (w/v) Protein A sepharose CL-4B (GE Healthcare) over night (4 °C). Proteins were separated on 10% (v/v) SDSpolyacrylamide gels followed by autoradiography for 4 weeks. Radiography was quantified using ImageJ v1.34s (http://rsb.info.nih.gov/ij/).

In Vivo Localization of GFP Fusion Proteins *Phaeodactylum tricornutum*

The BTSs of the *P. tricornutum* symbiont–specific ERAD homologs of Der1p (Pt_sDer1-1 Prot.-ID 31697,

bases 1–282; Pt_sDer1-2, Prot.-ID 35965, bases 1–348), of Cdc48 (Pt_sCdc48, Prot.-ID 50978, bases 1–345) and ubiquitin (Pt_sUbi, Prot.-ID 54323, bases 1–162) were amplified by standard reverse transcriptase (RT)–PCRs using specific oligonucleotides introducing a 5' *Eco*RI and 3' *NcoI* restriction site, using copy and genomic DNA from *P. tricornutum* as a template. PCR products were digested with *Eco*RI and *NcoI*, the plasmid pPhaT1 with *Eco*RI and *Hind*III and the green flourescent protein (GFP)-encoding fragment with *NcoI* and *Hind*III. All fragments were ligated and subsequently transformed into *Escherichia coli* MRF' in a single step. Fidelity of amplification and cloning was checked via sequencing of all constructs.

Phaeodactylum tricornutum transformations were performed as described in Apt et al. (1996). Analysis of transformed diatoms was performed with a confocal laser scanning microscope Leica TCS SP2 at room temperature in f/2 culture medium, using a PL APO 63x/1.32-0.60 oil Ph3 CS objective. GFP and chlorophyll fluorescence was excited at 488 nm, filtered with beam splitter TD 488/ 543/633, and detected by 2 different photomultiplier tubes with a bandwidth of 500–520 and 625–720 nm for GFP and chlorophyll fluorescence, respectively. Image processing was done using the LCS Lite software from Leica (Wetzlar, Germany).

Plasmodium falciparum

A fragment encoding the predicted signal peptide and TP of *P. falciparum* symbiont Der1 (Pf_sDer1-1, bases 1-416 of gene Pf14_0498) was PCR amplified from 3D7 genomic DNA with Phusion polymerase (New England Biolabs, Ipswich, UK) digested with *XhoI/AvrII* (NEB), and cloned into XhoI/AvrII digested pARL-STEVOR^{full} (Przyborski et al. 2005) to create pARLDBG. This construct was sequenced across the insert to verify fidelity of amplification and cloning.

Plasmodium falciparum strain 3D7 was cultured and ring stage parasites transfected as previously described (Przyborski et al. 2005). Selection was with 5 nM WR99210 (a gift of Jacobus pharmaceuticals, Princeton, USA) at 4 h posttransfection (Voss et al. 2006). Drug resistant parasites were first observed at day 22 posttransfection.

Infected erythrocytes were purified by gelafundin flotation, washed 3 times in serum free RPMI 1640, incubated with MitoTracker Orange CMTMRos and Hoechst33342 (both Molecular Probes), washed a further 3 times and mounted at 30% hematocrit under a cover slip on poly-llysine coated slides. Samples were viewed on a Leica TCS SP2 at room temperature (see above). Individual images were imported into Image J (http://rsb.info.nih. gov/ij), converted to 8-bit grayscale, subjected to background subtraction (rolling ball radius 25), and overlaid.

Protein Sample Preparation and Western Blotting

Phaeodactylum tricornutum cells were harvested at $2.500 \times \text{g}$ for 10 min and resuspended in protein extraction buffer (125 mM Tris/HCl pH 7.5, 1% sodium dodecyl sulfate [SDS] (w/v), 1% (v/v) TX-100, 0.5 mg/µl sodium desoxycholat [DOC] and proteinase inhibitor cocktail

[PIC]) and disrupted in repeated freeze and thaw cycles. After incubation at 4 °C with vigorous shaking for 1 h cell debris was pelleted by centrifugation ($20.000 \times g$, 15 min, 4 °C). Solubilized proteins were then precipitated from the supernatant with 10% (w/v) TCA in acetone, washed trice, and processed for SDS-Page in Laemmli sample buffer.

and processed for SDS-Page in Laemmli sample buffer. Trophozoite stage 3D7^{GFP} (Przyborski et al. 2005) and 3D7^{SDer1-1_BTS} infected erythrocytes were purified by gelafundin flotation. Erythrocytes were then lysed in 0.1% saponin/PBS, washed 3 times in PBS, subjected to repeated freeze/thaw cycles, and processed for SDS-Page in Laemmli sample buffer.

On a 15% polyacrylamide gel, $5-25 \ \mu g$ of protein was run, transferred to nitrocellulose membrane, blocked with PBS/5% skimmed milk powder, and probed with primary antibody. Mouse anti-GFP antibodies (Roche) were used 1:1.000 in PBS/5% skimmed milk powder. Membranes were washed in PBS and probed with horse radish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Clontech, St-Germain-en-Laye, France) 1:10.000 in PBS/5% skimmed milk powder.

Results

Nm-Encoded ER Proteins

In cryptophytes, 2 eukaryotic cytoplasms coexist adjacent to one another: the host cytosol and the symbiont's PPC, separated by the RER and PPM (Cavalier-Smith 2003). These 2 membranes are the entrance point for all nucleus-encoded proteins of the symbiont. Nucleusencoded plastid and PPC proteins enter the RER cotranslationally (Wastl and Maier 2000; Gould, Sommer, Kroth, et al. 2006). Sorting of these proteins from the secretory pathway is then accomplished by the TP of their BTS, which is exposed through cleavage of the signal peptide after membrane passage (Gould, Sommer, Kroth, et al. 2006). Proteins directed to either the PPC or the plastid stroma must however traverse the PPM. In order to identify possible candidates for a preprotein translocase of this membrane, we scanned the Nm data of G. theta (Douglas et al. 2001) for candidates. We detected 3 homolog factors known to be part of an ERAD system, which is surprising as the PPC lacks an endomembrane system apart from the functionally and topologically related Nm envelope (NE), which must potentially be regarded as a remnant ER. Amongst others, genes encoding homologs of the membrane proteins Hrd1p (ORF477), a RING-finger ubiquitin ligase (Bordallo et al. 1998), and Der1p (ORF201; Knop et al. 1996; Hitt and Wolf 2004) from yeast were detected in the Nm genome. Both are known to be essential components of the ERAD machinery for misfolded lumen proteins. Furthermore, we identified a C terminally truncated version of Ufd1, which, in association with Npl4 and Cdc48, constitute a cytosolic ubiquitin-dependent ERADassociated AAA-ATPase complex (Meyer et al. 2000). A homolog of Cdc48p is also present on the Nm genome as previously described (Douglas et al. 2001, table 1).

ORF201 Is a Functional Ortholog of Yeast Der1p

We performed a more detailed analysis of *G. theta* ORF201. This 23.5 kDa protein shares significant identity

 Table 1

 Nm-Encoded ERAD Components in Guillardia theta

Protein	Chromosome Number	Yeast Homologs	Function in ERAD
ORF201	1	Der1p	Cdc48-receptor and/or (part of the) translocon
ORF477	2	Hrd1p	RING-finger ubiquitin ligase (E3)
Ufd1	3	Ufd1p	Activator of Cdc48p
Cdc48	3	Cdc48p	AAA-type ATPase

to Der1p (21%) from *S. cerevisiae* (fig. 1), which is known to be involved in the translocation of misfolded soluble proteins from the ER into the cytoplasm for their subsequent degradation (Knop et al. 1996). Derlin-1 (the mammalian homolog of Der1p) was recently debated as either being a membrane-bound receptor for the Cdc48 complex (Romisch 2006) or part of the ERAD translocon itself (Lilley and Ploegh 2004; Ye et al. 2004). Like Der1p, ORF201 has 4 predicted TMDs, of which the first is predicted to be an ER signal anchor. Transcription of *orf201* was proven by RT–PCR (data not shown).

To investigate whether the *G. theta* Nm-encoded ORF201 is also a functional ortholog of the yeast Der1p (Degradation at the ER protein 1), we studied the degradation kinetics of CPY*, a soluble ERAD model substrate, in a *der1*-deficient yeast strain (Finger et al. 1993). CPY*, a mutated version of carboxy peptidase Y, is highly unstable due to its rapid degradation in ERAD, but accumulates in the *der1* mutant inside the ER (Knop et al. 1996).

Orf201 was episomally overexpressed in the $\Delta der1$ mutant. The half-life of newly synthesized [³⁵S]-labeled CPY* was observed in a time-dependent manner by immunoprecipitation of the remaining CPY* with a CPY-specific antiserum by pulse-chase experiments. Figure 2A shows that CPY* accumulates in the ER in $\Delta der1$ cells, whereas the overexpression of yeast *der1* (2µ *DER1*) restores CPY* degradation. Overexpression of *orf201* (2µ *201*) can partially restore degradation of the ERAD substrate. Quantification of autoradiographs pointed to a degradation of approximately 60% and 28% of CPY*, respectively, in 100 min postchase when overexpressing either Der1p or ORF201. This corresponds partly with results obtained

for the *der1*-like gene *R151.6* from *Caeanorhabditis elegans* (18% in 90 min; Hitt and Wolf 2004).

To confirm these results, we overexpressed the same constructs in the temperature-sensitive $\Delta der1/\Delta ire1$ double mutant and examined growth at a restrictive temperature (fig. 2*B*). Expression of *orf201* also suppresses the conditional lethality of the double mutant. Thus, ORF201 is a functional ortholog of Der1p and is able to facilitate the degradation of misfolded ERAD substrates in yeast by directly or indirectly relocating them into the cytosol.

Two Independent ERAD(-like) Machineries in Chromalveolates with 4 Membrane–Bound Plastids

Other than the *orf201*, a gene emerged in our expressed sequence tag (EST) library encoding a second, but host-specific *der1*-like protein (Derlin; *G. theta der11*). As this host Derlin lacks any topogenic signals apart from the required one for successful insertion into the ER membrane, our data indicate that 2 independent ERAD(-like) systems coexist in cryptophytes.

To determine whether the ORF201-based ERAD-like machinery is exclusive to cryptophytes (possibly due to the unique morphology of the secondary symbiont) or present in other chromalveolates, we screened the genome and EST databases of 2 chromalveolates, that of the raphid, pennate diatom P. tricornutum CCAP1055/1, and of the apicomplexan malaria pathogen P. falciparum (strain 3D7) for symbiont-specific "ERAD-components." In cryptophytes, the membrane proteins ORF201 and ORF477 are encoded in the Nm genome. Thus, these proteins should be synthesized and inserted into their target membrane (PPM, NE, or outer plastid envelope membrane [OM], see Discussion) from inside the PPC. Unlike the situation in cryptophytes, these proteins should-if present in other chromalveolates-be nucleus encoded as their symbionts all lack a Nm. Accordingly, they should posses BTS similar to that of nucleus-encoded PPC proteins of cryptophytes (Gould, Sommer, Hadfi, et al. 2006; Gould, Sommer, Kroth, et al. 2006), which is required for entering the PPC-homologous region between the first and last membrane pair of plastids/ apicoplasts of diatoms and apicomplexa.



FIG. 1.—Amino acid alignment of yeast Der1p and ORF201. TMDs are indicated in light gray (TMHMM). Identical residues are shaded in black and similar ones in dark gray. *Guillardia theta* ORF201 and *Saccharomyces cerevisiae* Der1p share 21% sequence identity.









FIG. 2.—Complementation experiments in yeast. (A) Pulse-chase analysis of CPY* degradation in $\Delta der1$ cells (W303-CD) expressing pRS424TDH, yeast Der1p (pRS424TDH $der1/2\mu$ DER1), or Guillardia ORF201 (pRS424TDH $orf201/2\mu$ 201). Orf201 is able to rescue the yeast der1 partially. Each graph represents the average of at least 3 individual experiments; standard deviation is indicated by error bars. (B) Growth of the temperature-sensitive $\Delta der1/\Delta ire1$ double mutant (YRH110) expressing the same plasmids as in (A) assessed on SD/Trp medium for 3 days at 25 °C or 37 °C.

Several genes encoding ERAD(-like) components with and without a BTS were identified in the genomes of *P. tricornutum* and *P. falciparum*, indicating that these organisms encode, in addition to the host's ERAD machinery (proteins without a BTS) symbiont-specific ones as well. All identified genes, ESTs or gene models are listed in table 2. All together, 4 genes encoding Derlin proteins were identified in both organisms, 2 host-specific and 2 symbiont-specific ones. In analogy to current nomenclature, we termed them Der1-1 and Der1-2, respectively. As they were functionally not characterized, we can not deduce from our data if these are real Der1 proteins or rather homologs of the closely related Dfm1p (Der1-family member) protein, which was recently shown to function together with Cdc48p in ER homeostasis (Sato and Hampton 2006). In P. tricornutum, the symbiont-specific Derlins as well as the Cdc48 cofactor Ufd1 are encoded as preproteins with a BTS already "in frame" with the ORF. The BTS from the symbiont-specific homolog of Cdc48 is encoded on exon I, the mature protein on exon II, separated by an intron with typical spliceosomal intron borders. In P. falciparum, both Derlins and the Cdc48 homologs are annotated as putative apicoplast proteins in PlasmoDB (Kissinger et al. 2002). As Derlins are less conserved than other components of the ERAD machinery, the *P. falciparum* symbiont-specific Derlins were annotated as hypothetical proteins. Two genes encoding Ufd1 were detected in *P. falciparum*. One of these bears a putative apicoplast-targeting signal in the predicted 5' untranslated regions of the gene, which is spliced in frame directly before the gene (Spork S, Przyborski JM, unpublished data).

Further components which can be attributed to a symbiont-specific ERAD(-like) system were identified as being encoded in *P. tricornutum*: ubiquitin, Ubc6 a membrane-bound ERAD-associated ubiquitin conjugation enzyme (E2), and the ubiquitin activation enzyme Uba1 (E1). The latter was also identified in *P. falciparum*. A symbiont-specific homolog of Hrd1, shown to be present in the Nm genome of *G. theta* (ORF477), has not been identified thus far.

To test the targeting properties of *P*. *tricornutum* and P. falciparum presequences from identified symbiontspecific ERAD components, we exemplarily fused the topogenic signal of each orf201 homolog to GFP, and expressed it in the homolog system (fig. 3). As expected, the GFP-fluorescence of the symbiont-specific Der1-1_BTS of *P. tricornutum* (clone $1055/1_{GFP}^{sDer1-1_BTS}$) was observed in a distinct dot, tightly associated but not colocalized with the plastid autofluorescence (fig. 3A). Recently, we have shown that such a localization, formerly addressed as a "blob-like" structure (Kilian and Kroth 2005), is located in a region equivalent to the cryptophyte PPC as it always occurs within the 2 spearhead-shaped, opposing plastid halves (Gould, Sommer, Kroth, et al. 2006). Accordingly, BTS of the P. falciparum symbiont Der1-1 the $(3D7_{\text{GPP}}^{\text{sDer1-1-BTS}})$ is able to direct GFP to the apicoplast (3C). Staining with Hoechst and MitoTracker (see Material and Methods) proves that the GFP expression does not display a nuclear or mitochondrial localization. Furthermore, western blot analyses with α -GFP antibodies reveal processing of the TPs of both ORF201 homologs (fig. 3B and D).

Finally, the BTS of a number of further symbiontspecific ERAD homologs from *P. tricornutum* like Der1-2, Cdc48, or ubiquitin (fig. 4) as well as a PPC-specific Hsp70 (Prot.-ID 55890, Gould, Sommer, Kroth, et al. 2006), could also be shown to direct GFP to the diatom's PPC.

Discussion

An ERAD System in the Secondary Symbionts of Chromalveolates

The endoplasmic reticulum is one of the most diverse compartments inside eukaryotic cells. Among its multiple functions in protein sorting and modification, its own quality control mechanism for secretory proteins is most remarkable. Misfolded secretory proteins are exported from the ER and degraded in the cytosol via ERAD. Substrate relocation is mediated by an ubiquitin-dependent translocase, potentially composed of Sec61 and/or Der1 and multiple accessory proteins (Romisch 2005).

In cryptophytes, we have identified, beside the host's own ERAD machinery, a second one, located in the PPC of

Table 2
List of All So Far Identified ERAD(-like) Components Encoded in the Genomes of Guillardia theta, Phaeodactylum tricornutum
and Plasmodium falciparum

	Cryptophytes Guillardia ^a		Heterokontophytes Phaeodactylum ^b		Apicomplexa Plasmodium ^a	
Protein Sec61a						
	Host specific AM183806	Symbiont specific	Host specific 56443	Symbiont specific ND	Host specific CAD52584	Symbiont specific ND
Uba1 (E1) Ubc6 (E2)			54246 54754 56437	ND 54460 56431	CAD51861 AAN36335 ND	ND CAD52471 ND
Hrd1 (E3) Ubi	_	CAC27064	49801 56440	ND 54323	AAN36828 AAN36206	ND ND
Der1-1 Der1-2	CAK12751	AAK39810	46966 37614	31697 35965	AAN37266 AAN35514	AAN37111 CAB38986
Cdc48 Npl4	_	AAK39773	21083 56433	50978 ND	CAG25009 CAD51442	CAD50861 ND
Ufd1 Ufd2 (E4)	_	AAF24006	44372 35755	49319 ND	AAN36790 CAD51109	ABK41123 ND
Png1 Rad23 Hsp70	 CAD2478	 	50113 43564 54019	ND ND 55890	ND AAN35312 CAD51185	ND ND ND

NOTE.-ND, not detected in the available databases; ---, no genomic or EST data existing thus far.

^a Gene/gene models are or will be available under their accession number or protein ID at NCBI.

^b Gene/gene models are or will be available under their accession number or protein ID at Joint Genome Institute.

the secondary symbiont. As an example, we studied ORF201, the G. theta Nm-encoded homolog of Der1, a key player in ERAD. Further homologs of ERAD components identified were Cdc48, Ufd1, and Hrd1 (ORF477; table 1). We demonstrated that ORF201 is a functional ortholog of Der1p from yeast as shown by complementation experiments, in which ORF201 was able to rescue an $\Delta derl$ -deletion mutant by facilitating the degradation of CPY*, a soluble ERAD model substrate (fig. 2). Thus, our findings strongly indicate that the secondary symbiont of cryptophytes expresses at least a partial ERAD-like system of its own. Moreover, we can show that this system is not exclusive to cryptophytes, as other secondarily evolved organisms with a red algal symbiont (like *P. tricornutum* and P. falciparum) also possess genes encoding components of this machinery. These genes are nucleus encoded in such organisms, and gene products must be transported across the 2 outermost membranes of the symbiont. Accordingly, the symbiont-localized factors are endowed with PPC-specific BTSs (without a phenylalanine at position +1) of the TP in diatoms), which were detected parallel to the BTS-less host copies in the P. tricornutum and P. falciparum databases (2 Derlins, Cdc48, Ufd1, and Uba1). An exemplary test for the in silico prediction of the BTS from endogenous ORF201 homologs demonstrated a localization in the region corresponding to the PPC of *P. tricornutum* and to the apicoplast of P. falciparum (fig. 3A and C). The BTS of a number of further nucleus-encoded symbiont-specific ERAD(-like) components (e.g., sCdc48 and sUbi) could also direct GFP to the PPC of P. tricornutum (fig. 4).

Our complementation experiments of the yeast mutant show that ORF201 assumes the same topology as the yeast Der1p (with the N and C termini on the cytosolic side of the membrane) and operates in the same direction (lumen to cytosol). Therefore, our experiments show that ORF201 is capable of facilitating the transport of unfolded proteins from a luminal to a plasmatic compartment. Given to the peculiar morphology of the cryptophytes' secondary symbiont, 3 possible destinations exist a for symbiontspecific ERAD-like system represented by ORF201 (and ORF477): the NE—potentially constituting the symbiont ER, the PPM, which separates the PPC from the host RER-lumen, and the OM. As all chromalveolates seem to have a common, monophyletic origin (Cavalier-Smith 1999, 2003; Harper et al. 2005), a conservation of this system, also at the functional level, would be expected.

A localization in the OM seems to be most unlikely as this would imply that the ERAD-like system would have to be involved in protein transport processes for which no precedent currently exists (retrotranslocation from the intermembrane space of the plastid into the cytoplasm).

We have shown that symbiont-specific Derlins also exist in organisms with secondary symbionts without a Nm retained in their PPC, thus a destination in the NE seems—although evolutionary being the original location for this system—impractical. Nevertheless, endomembranes have been discussed to be present in at least the PPC of heterokontophytes, supposedly relics of the periplastid reticulum (PPR) (Gibbs 1979), and a ERAD-like machinery present in this PPR, could function in regulatory protein degradation events of the symbiont.

It was recently shown that some members of the Derlfamily function together with Cdc48 in roles distinct from ERAD, such as ER homeostasis (Sato and Hampton 2006). Furthermore Cdc48, as well as its involvement in multiple different other cellular processes, also mediates homotypic fusion of the ER membrane (Latterich et al. 1995). A localization in the NE or PPR might, therefore, be more likely than it appears at first sight, and could imply a more general function of this system in processes of organelle assembly and maintenance, for example, in PPM biogenesis. Nevertheless, a PPR was identified in some secondary evolved organisms, which may be the localization of the ERAD-like system in these secondary symbionts.



FIG. 3.—In vivo localization of the *Phaeodactylum tricornutum* and *Plasmodium falciparum* ORF201-homolog presequences. (A) The bipartite topogenic signal of the *P. tricornutum* (strain CCAP1055/1) ORF201 homolog sDer1-1 (1055/1^{sDer11_BTS}, ~37 kDa in size) drives GFP into the PPC homolog region of the secondary symbiont (in green, plastid autofluorescence is in red) and (*B*) is cleaved after import, leading to a ~28 kDa fragment (wild type, 1055/1; cytosolic expressed GFP, 1055/1_{GFP}). (*C*) Localization of the symbiont-specific Der1-1 BTS–GFP fusion protein in *P. falciparum* 3D7 blood stage parasites (3D7^{SDer1-1_BTS}, in green) additionally stained with MitoTracker (in red) and Hoechst (in blue). The typical "dot" pattern of apicoplast targeted GFP is clearly evident. (*D*) The *P. falciparum* sDer1-1_BTS construct (3D7^{SDer1-1_BTS}, ~43 kDa in size) is processed after import (~30 kDa), A GFP degradation product (~GFP) is also seen, as has previously been reported (Waller et al. 2000), wild type (3D7) and cytosolic expressed GFP (3D7_{GFP}, ~27 kDa) as controls. Scale bar represents 4 µm.

A further possible localization for the system is in the former plasma membrane of the symbiont, the PPM. As a primary machinery for degradation of misfolded proteins, which failed to pass the protein-folding quality control of the ER, this system could contribute to the elimination of these proteins from the host ER. Although it is feasible that cryptophytes could use degraded proteins as a source of amino acids for catabolic processes within the PPC, the apicomplexan outer apicoplast membrane seems not to be continuous with the host's ER (van Dooren et al. 2001) making such a function highly unlikely if we hypothesize an evolutionary conserved function in all organisms.

ERAD itself is as diverse as its substrates. In fact, it seems that no 2 substrates have the same protein requirements for degradation, and different designated pathways—depending on the state of misfolding—exist in parallel in both yeast and mammals, converging only in the dependence on the Cdc48 complex in the cytosol (Meusser et al. 2005; Romisch 2005). Recent work has revealed a clearer picture of the proteins required for the degradation of various substrates (Carvalho et al. 2006). Interestingly, of all the proteins associated with the ERAD system, it seems that Der1 and Hrd1 (together with Hrd3 and Usa1) are most important for the degradation of soluble luminal proteins and membrane proteins with luminal



FIG. 4.—In vivo localization of *P. tricornutum* bipartite presequences fused to GFP. The BTSs of the endogenous *P. tricornutum* symbiont-specific ERAD(-like) homologs of Cdc48 ($1055/1_{GFP}^{Scle48_BTS}$), Der1-2 ($1055/1_{GFP}^{Scle48_BTS}$), and ubiquitin ($1055/1_{GFP}^{SUbel1_BTS}$) direct GFP (in green) into the space between the 2 inner and outermost membranes of the complex plastid, indicated by the typical dot-like fluorescence of the diatom's PPC. Plastid autofluorescence is in red. Scale bar represents 5 µm.

lesions. As the PPC and the plastid have to be provided with thousands of proteinaceous factors (Martin et al. 2002; Cavalier-Smith 2003; Gould, Sommer, Kroth, et al. 2006), which have to be kept unfolded and soluble until they reach their final destination within the symbiont (Gould, Sommer, Kroth, et al. 2006), a role of the symbiont-specific ERAD machinery in this matter can also be proposed. As the initial stages of ERAD represent the only known mechanism by which proteins can be exported from the ER into the cytosol, the idea of a Der1-based preprotein import machinery for the PPM is quite attractive. This would not be without precedence, as the peroxisomal import machinery was also derived from components of the ERAD machinery (Gabaldon et al. 2006).

A New Preprotein Import Model for the Periplastid Membrane

Almost 2 decades of research into protein transport across the multiple membranes of chromalveolate plastids have advanced our understanding of how proteins traverse the outer and innermost membrane of 4 membrane–bound plastids (Wastl and Maier 2000; Douglas et al. 2001; McFadden and van Dooren 2004; Kilian and Kroth 2005; Gould, Sommer, Kroth, et al. 2006; Tonkin et al. 2006). Despite this, however, so far only models exist to explain the transport mechanism at the second outermost membrane. When Cavalier-Smith proposed a monophyletic origin of all secondary symbionts of red algal origin (Cavalier-Smith 1999), he grouped them into the clade chromalveolates. Having evolved in the progenitor of these organisms,



FIG. 5.—An ERAD-derived preprotein import model for the PPM of cryptophytes. The host ERAD machinery is in blue, the symbiont-specific Derlin-based preprotein import machinery in red and the TP of cargo proteins is in green. Host-/symbiont-specific ubiquitins are indicated by blue or red dots. Nu, host nucleus; Mi, mitochondrion; IMS, plastid intermembrane space; TP-R, TP receptor.

a general mechanism of protein import at the PPM can be postulated. With the identification of a symbiont-specific ERAD-like system in cryptophytes, heterokontophytes, and apicomplexans, we provide the first molecular evidence for a conserved machinery able to fulfill all criteria for a preprotein translocase present in the PPM (see below).

In chromalveolates with secondary symbionts located within the RER, the ERAD and ERAD(-like) systems coexist next to each other. Hence, the symbiont-specific one, if really relocated to the PPM to direct preprotein import into the PPC, would have to differ significantly in its substrate specificity and chaperone requirement from the "classical" ERAD machinery. Substrates for ERAD are recognized by a distinct modification of their N-glycans (Helenius and Aebi 2004), resulting from prolonged chaperone association. We have recently shown that plastid and PPC proteins are sorted from secretory proteins by the TP of their BTS (Gould, Sommer, Hadfi, et al. 2006; Gould, Sommer, Kroth, et al. 2006). Hence, the TP alone or complexed with mediating proteins would have to serve as the ligand for an import receptor (TP receptor), which guides the cargo proteins to the postulated translocon of the PPM (translocator of the periplastid membrane [TOP]).

The exact nature and identity of the classical ERAD translocon (long postulated to be essential for ERAD of soluble proteins), remains unclear in all systems investigated. Earlier models based on data from the yeast system, suggested that Sec61 constitutes the core of the ERAD translocon (Romisch 2005). In contrast, our data suggest that Sec61 may play a nonessential role in ERAD-mediated protein translocation as neither PPM- nor NE/PPR-targeted homologs of this protein were identified in our searches. This would also coincide with recent discussion that the Sec61 protein-conducting channel seems not to be qualified for its proposed function in ERAD (Tsai et al. 2002; Meusser et al. 2005) as some substrates are not necessarily dislocated in an totally unfolded state (Tirosh et al. 2003) and might be expected to clog the hourglass-shaped pore of the Sec61 translocon. In the case of SecYEG, the archeal homolog of Sec61 channel, it was estimated that the translocon only allows the passage of proteins with an maximum diameter of approximately 10-12 Å, whereas, some ERAD substrates had a calculated diameter of at least double this size. Nevertheless, other studies estimated a 4-fold to 5-fold pore size expansion during cotranslational protein import (Meusser et al. 2005), and ribosomes bound to the protein-conducting channel have been shown to decrease

the degradation of at least 1 ERAD substrate (Schmitz et al. 2000).

As not all ERAD substrates seem to depend on Sec61, the multispanning membrane proteins Der1 and/or Hrd1 have also been suggested to be putative channel or transient pore building proteins (Meusser et al. 2005). Both are known to be essential for the degradation of at least a subset of proteins (Taxis et al. 2003) and especially required for degradation of soluble and membrane-bound ERAD substrates with luminal lesions (Carvalho et al. 2006). In any case, no second Sec61 or another symbiont-specific Sec subunit was identified in chromalveolates. Hence, a Sec61-independent function is likely for the symbiont-specific "ERAD", irrespective of its localization inside the PPC. Der1 alone or in association with the Hrd1 (a homolog of which was found in cryptophytes, ORF477) may facilitate the dislocation of preproteins into the PPC, building either a homo or heteromeric channel or a transient pore. How ORF201 and ORF477 insert into the PPM, and what topology they assume remains uncharacterized. Knowledge of the topology has immediate consequences for the transport direction of the translocon. As mentioned before, 2 points can be concluded from our ORF201 complementation experiments in yeast. First, Der1p and ORF201 share the same topology in the target membrane and secondly, both act to translocate proteins in the same direction (lumen to cytosol).

Relocation of preproteins from the RER can be expected to be facilitated by a PPC-located AAA-ATPase complex. In ERAD, the various different pathways converge in being dependent on the cytosolic ATPase CDC48/p97 and its ERAD-specific cofactors Npl4 and Ufd1, which extracts substrates from the membrane (Ye et al. 2004; Neuber et al. 2005; Schuberth and Buchberger 2005). For this, proteins are ubiquitinylated upon export. Ubiquitinylation is carried out either by the ubiquitin-ligase complex Hrd3/Hrd1 or ubiquitin-ligase Doa10 (Ismail and Ng 2006), together with the ubiquitin-conjugating enzymes Ubc7/Cue1 or Ubc6 and the ubiquitin activation enzyme Uba1 (Biederer et al. 1997; Lenk et al. 2002). As well as the Nm-encoded factors in cryptophytes, genes encoding symbiont-specific homologs of Cdc48, Ufd1, and Uba1 were identified in the genomes of *P. tricornutum* and *P.* falciparum, and symbiont-specific copies of Ubc6 and ubiquitin are at least present in the *P. tricornutum* genome, giving credence to the idea that preprotein import might be mechanistically similar to ERAD substrate relocation.

As mentioned before, the symbiont-specific Ufd1homologs are C terminally truncated compared with the yeast homolog. Both the Npl4- as well as the CDC48binding sites are missing. Hence, if these "Ufd1" proteins are part of an Der1-derived TOP-translocon, another specific factor would have to be present (not shown in fig. 5) to recruit Cdc48 via the truncated UFD1 to the ubiquitinylated cargo proteins emerging from the postulated export channel. It should be noted that the truncated Ufd1 is conserved among the chromalveolates and that Cdc48 is generally recruited to its different cellular functions by specific cofactors, such as p47 or Npl4-Ufd1 in other systems (Meyer et al. 2000; Sato and Hampton 2006). Furthermore, no symbiont-specific Npl4 was detected in the diatom or apicomplexan genome thus far. After export, the ubiquitin-chain assembly factor Ufd2p (E4) elongates the ubiquitin chains of oligoubiquitinylated ERAD substrates, thereby determining their fate (Richly et al. 2005). Ubiquitin residues are attached via the carboxyl-residue of their C-terminal glycine to the Lys48 of the preexisting ubiquitin. Lys48-multi-ubiquitin chains are specifically recognized by the proteasome via Rad23p (fig. 5). Interestingly, the detected putative symbiont-specific ubiquitin of *P. tricornutum* lacks this lysine at position 48 (Lys to Gln exchange), effectively preventing its poly-ubiquitinylation. Additionally, no symbiont-specific Ufd2 or Rad23 were identified, neither in the Nm genome of *G. theta* nor in the genomes of *P. tricornutum* and *P. falciparum* (table 2).

Having arrived in the PPC, translocated proteins have to be directed to the plastid envelope for subsequent transport steps or, if they are PPC-residents, are processed and folded. We postulate that in both cases they are initially received by a PPC Hsp70, a copy of which is encoded in the cryptophytes' Nm and as a preprotein also in the *Phaeodactylum* genome (table 2). Whereas, PPC-residential proteins might fold, a guidance complex such as that suggested for higher plants might carry the plastid proteins to the translocon of the outer envelope membrane (Jarvis and Soll 2002) (fig. 5).

Concluding Remarks

By data mining, in vitro and in vivo tests, we provide evidence that besides the host's own ERAD machinery, a second symbiont-specific ERAD-like machinery is present in the secondary symbiont of cryptophytes. This system is not unique to cryptophytes, but rather present in all investigated chromalveolates with 4 membrane–bound plastids. We propose that this machinery was relocated from the symbiont's ER to the PPM and was reutilized as the long hypothesized and sought translocator for nucleus-encoded plastid/ PPC preproteins of the PPM. If so, we provide the first experimentally accessible model to study this issue. Future experimental efforts are required to validate its function and exact localization within the PPC of chromalveolates.

Supplementary Material

New and relevant EST data was deposited at NCBI under CAK12751.

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