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The chimeric origin of the cardiolipin biosynthetic pathway in the Eukarya domain

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ABSTRACT

Cardiolipin (CL) and phosphatidylglycerol (PG) are the main anionic phospholipids present in the Eukarya and Bacteria domains. They participate in energy transduction by activating and stabilizing the components of the oxidative phosphorylation machinery. Experimental evidence shows that they are synthesized by two different mechanisms which indicate that both pathways evolved convergently. Former studies on the lipid composition of archaeal membranes showed the absence of CL in these organisms, consequently, restricting it to the Eukarya and Bacteria domains. Interestingly, recent studies have demonstrated that both CL and PG are present as constitutive components of membranes of the haloarchaea group. However, this scenario complicates the analysis of the evolutionary origin of this biosynthetic pathway. Here I suggest that a phospholipid biosynthetic pathway in Eukarya probably arose from a chimeric event between bacterial and archaeal enzymes during the endosymbiosis event. Phylogenetic analyses support the different evolutionary origin of the enzymes comprising this pathway in bacteria and Eukarya. Based on protein domain analyses, orthologous proteins in the Archaea domain were identified. An integrative analysis of the proteins found demonstrates that CL biosynthesis in major clades of the Eukarya domain originated by chimerism between the bacteria and archaea pathways. Moreover, primary and secondary endosymbiontic events in plants and chromoalveolata respectively, reshaped this pathway again. The implications and advantages that these new enzymatic orders conferred to the Eukarya domain are discussed.

1. Introduction

Anionic phospholipids such as phosphatidylglycerol (PG) and cardiolipin (CL) are key components of cellular membranes, where they have important roles in several cellular processes such as energy transduction, stress response, participation in the mechanism of translation coupled to transcription (transertion) in bacteria and the stabilization, maintenance and segregation of mitochondrial DNA [1–4]. CL was believed to be restricted to the Bacteria and Eukarya domains because early evidence ruled out the existence of this phospholipid in archaeal membranes. Since PG has been demonstrated to be present in the three domains of life, it was believed that CL spread through eukaryotic cells during the endosymbiotic process from the bacterial endosymbiont [5]. This idea is being challenged based on the more recent discovery of CL in several archaeal species [6].

Apparently, PG and CL biosynthesis evolved convergently in bacteria and eukaryotes since they are synthesized by two different enzymatic pathways (see Fig. 1). In the bacterial pathway, glycerol 3-phosphate is condensed to a molecule of CDP-DAG, producing phosphatidyglycerol phosphate (PGP), which is dephosphorylated to produce PG. It is then condensed to another molecule of PG to produce CL. On other hand, in the eukaryotic pathway, glycerol 3-phosphate is transferred to a CDP-DAG molecule to produce PGP, as in bacteria. However, after dephosphorylation of PGP, PG is condensed to another CDP-DAG molecule producing CL, thus differing in this step in the bacterial substrates used for synthesis [7]. As stated before, the enzymatic mechanisms differ between domains: in bacteria, the first enzyme, phosphatidylglycerol phosphate synthase (Pgs_T), belong to the transferase class (E.C. 2.7.8.5), whilst in Eukarya the enzyme is a hydrolase (E.C.3.1.4.4) from the phospholipase D (PLD) family (Pgs_D). Conversely, cardiolipin synthase (Cls_D) activity in bacteria belongs to the hydrolase class (PLD) whilst the eukaryote (Cls_T) enzyme belongs to the transferase class. These differences in the position of each enzymatic step indicate that CL biosynthesis could have evolved independently in each domain. Interestingly, several versions of this pathway have been observed in eukaryotes: whilst in Kinetoplastid and Apicomplexan organisms both enzymes are phospholipases D, in plants both enzymes are CDP transferases. Moreover, recent evidence *in silico* has demonstrated the presence of a bacterial-like phosphatidylglycerol phosphate synthase together with evidence of CL in several species of archaeas [8–10]. This intricate diversity of pathways for CL biosynthesis has been poorly studied; the only study was restricted to uncovering the evolution of Cls and CL-remodeling enzymes (a process restricted to the Eukarya

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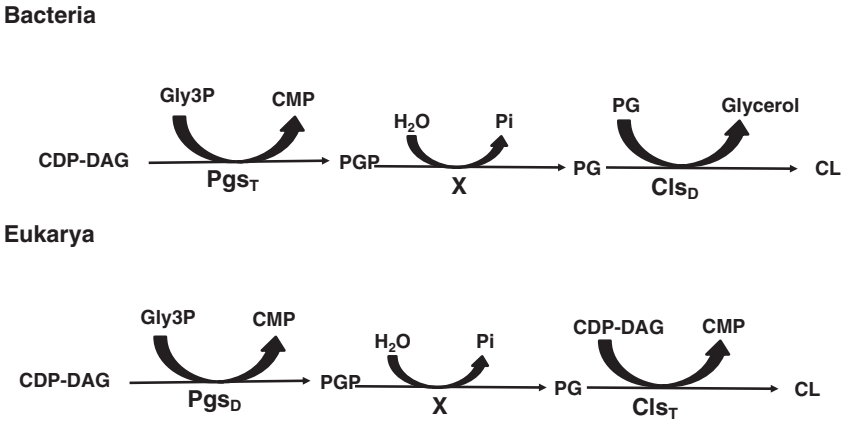

Fig. 1. Cardiolipin biosynthesis in the Bacteria and Eukarya domain. Although the first enzyme common for both domains uses the same substrates, the enzymatic mechanism varies between them. The bacterial enzyme is a CDP-DAG transferase whilst the eukaryote enzyme is a PLD. On the other hand, the Cls is a PLD in bacterial whilst a CDP-DAG transferase in Eukarya. The archeal pathway is believed to be identical to the bacterial. Pgs, phosphatidylglycerol phosphate synthase; Cls, cardiolipin synthase; CDP-DAG, cytidine diphosphate-diacylglycerol; PGP, phosphatidylglycerol phosphate; PG, phosphatidylglycerol; CMP, cytidine monophosphate. The X symbol represents a poorly conserved enzyme necessary for the hydrolysis of the ester bond between glycerol and phosphate from the head group.

domain). This study concluded that eukaryal Cls activity evolved from two endosymbiotic events, one giving rise the first eukaryotic common ancestor (FECA) with a PLD activity and the second one from a secondary endosymbiont replacing the FECA Cls with a transferase enzyme [5].

In this report, the evolution of cardiolipin biosynthesis was reanalyzed together with orthologous proteins found in the Archaea. I suggest that CL biosynthesis in the Eukarya domain rose by a chimeric mechanism involving both archaeal and bacterial proteins. Subsequent endosymbiotic events provided the basis to further diversify this pathway into the Eukarya domain. This evolutionary exchange had important repercussions in the subsequent evolution and specialization of several mitochondrial features.

2. Methods

2.1. Identification of sequences from CL synthase and PGP synthase

A phylogenetic distribution of the four enzymes through the three domains was retrieved from STRING v.9.1 [11]. Aminoacid sequences for phosphatidylglycerol phosphate synthase (Pgs) and cardiolipin synthase (Cls) were identified by Domain Enhanced Look up Time accelerated (DELTA)—BLAST from the NCBI server using *Saccharomyces cerevisiae* and *Escherichia coli* sequences as queries. All proteins selected had a maximum *E*-value $< 10^{-15}$. Same results were obtained using *Bacillus subtilis and Homo sapiens* sequences as queries. For clarity, a subscript D means that the enzyme has a phospholipase D mechanism whilst a C it is a transferase. 143 non-redundant sequences for phospholipase D enzymes (Pgs_D and Cls_D) and 140 for transferase enzymes (Pgs_T and Cls_T) were selected for phylogenetic reconstruction.

Ancestral sequences of the Cls_T and Pgs_T groups were acquired after phylogenetic reconstruction of the respective tree using the ancestral reconstruction function implemented in Mega 6 software [12]. Multiple alignments between those sequences and the Cls from *S. coelicolor* were aligned with ClustalW.

Transmembrane helix topology of PLD sequences was performed with the toppred program implemented in the Mobyle 1.5 portal developed by the Institute Pasteur Biology IT Center and the *Ressource Parisienneen Bioinformatique Structurale* (mobyle.pasteur.fr/cgi-bin/portal.py#welcome) [13].

2.2. Phylogenetic analysis

Selected sequences were aligned with MUSCLE (v3.7) without curation. Sequences causing aberrant alignments were removed from posterior analysis. Trees were inferred using the maximum likelihood method with the Mega6 program. The Jones–Taylor–Thornton method was used as substitution matrix. Statistical significance was calculated by the bootstrapping method using 100-pseudoreplicas [12].

3. Results

3.1. Taxonomical distribution of CL biosynthetic enzymes

A first search for the occurrence of all Pgs_D, Pgs_T, Cls_D and Cls_T sequences showed that Pgs_T was the only enzyme confidently distributed between, Bacteria and Archaea domains, but absent in Eukarya. All other enzymes were present in just one domain. For Archaea, proteins homologous to Cls_D and Pgs_D present homology to phospholipases D with unknown functions (Table 1). Interestingly, eukaryote Pgs_D presented poor similitude to bacterial Cls_D. Since these preliminary results show that Pgs_D was absent in Bacteria, questions arise: How did this enzyme appear in Eukarya? Would it be possible for Pgs_D to be related to archaeal phospholipases D? A refined search based on conserved domains was performed to answer these questions. Each enzymatic activity was analyzed individually.

3.2. CDP-DAG transferases

Previous *in silico* studies identified several proteins in methanoarchaea as homologous to bacterial Pgs_T [14]. However, experimental evidence has not corroborated the catalytic activity of those proteins as with other members of this family, including the archeal phosphatidylinositol synthase (Pis) [15]. Thus, Cls_T from *S. cerevisiae* and *H. sapiens*, and Pgs_T from *E. coli* and *Methanosarcina barkeri* (an archaea) were used as queries.

Table 1
Domain distribution of the enzymes involved in CL biosynthesis. Phosphatidylglycerol phosphate synthase (Pgs) and CL synthase (Cls).

Protein	Bacteria	Achaea	Eukarya
PgsD	○	?*	●
PgsT	●	●	○
ClsD	●	?*	○
ClsT	○	?	●

Note: D—phospholipase D mechanism, C—CDP-DAG transferase mechanism, ○—absent, ●—present, ?—unknown. *—Protein similar to phospholipase D. Pgs, phosphatidylglycerol phosphate synthase. Cls, cardiolipin synthase.

I recovered 140 sequences with a maximum *E*-value of 10^{-15}. The topology of the reconstructed tree clearly presented a monophyletic distribution for the sequences within each domain (Fig. 2). This means that all eukaryotic Pgs present a common ancestor and same stands for bacterial Cls andPgs from archaeal proteins.

Within domains, archaea present the most consistent topology, clearly distinguished between Euryarchaeota (*i.e. Haloferax volcanii*) and Crenarchaeota (*i.e. Sulfolobus islandicus*) sub-domains. Several sequences recovered were from organisms where PG has been observed as a constituent of membranes. In general, PG is widely used for archaeas for the biosynthesis of two characteristic archeal lipids: phosphatidylglycerol methyl ester and phosphatidylglycerol sulfate [16]. These sequences together form the outgroup of the cluster integrated by the bacterial and eukaryal sequences. As expected, eukaryal Pgs and bacterial Cls cluster together forming a clear monophyletic group. Some exceptions are observed in some sequences for the representatives of land plants, red and green algae, which cluster within the bacterial Cls group. Interestingly, these eukaryotes present sequences represented in both CDP-DAG transferase groups, indicating that Archaeplastids replaced one or both enzymes in mitochondrial CL biosynthesis during the primary endosymbiosis event leading to chloroplast development. Two findings highlight the origin of an enzyme from the plastidic ancestor of the chloroplast. First, Opisthokonta (Fungi and Metazoa) and Amoebozoa clades, which have single mitochondria, present a Cls exclusively with a transferase mechanism. Second, in Archaeplastids, PG is supplied to chloroplasts and mitochondria by only one enzyme [17]. Thus, Pgs are expected to be targeted to both compartments, as in *Chlamydomonas reinhardtii*, or to follow the strategy of land plants such as *Arabidopsis thaliana*, where a set of paralogues with redundant function are observed [18]. However, each isoform is targeted to chloroplasts and mitochondria. Thus, this solves the problem of providing PG to both organelles. Many genes derived from plastids follow this behavior such as fructose 1,6-bisphosphate aldolase or glyceraldehyde 3-phosphate dehydrogenase, which replaced the host proteins [19,20].

Notable is the presence of a transferase in the Rhizaria *Paulinella chromatophora*. Since no single enzyme was recovered from the Rhizaria super-group, this indicates that during secondary or tertiary endosymbiont events giving rise to this super-group, both enzymes were lost. However, the relatively recent evolutionary acquisition of a cyanobacterial endosymbiont by *P. chromatophora* shows that this protein was horizontally transferred by its symbiont, related to the cyanobacteria *Prochlorococcus marinus* or *Synechococcus* sp [21].

Another feature observed in this phylogeny is the presence of actinobacterial sequences localized deep inside in the eukaryote tree. Experimental evidence has demonstrated that several actinobacteria

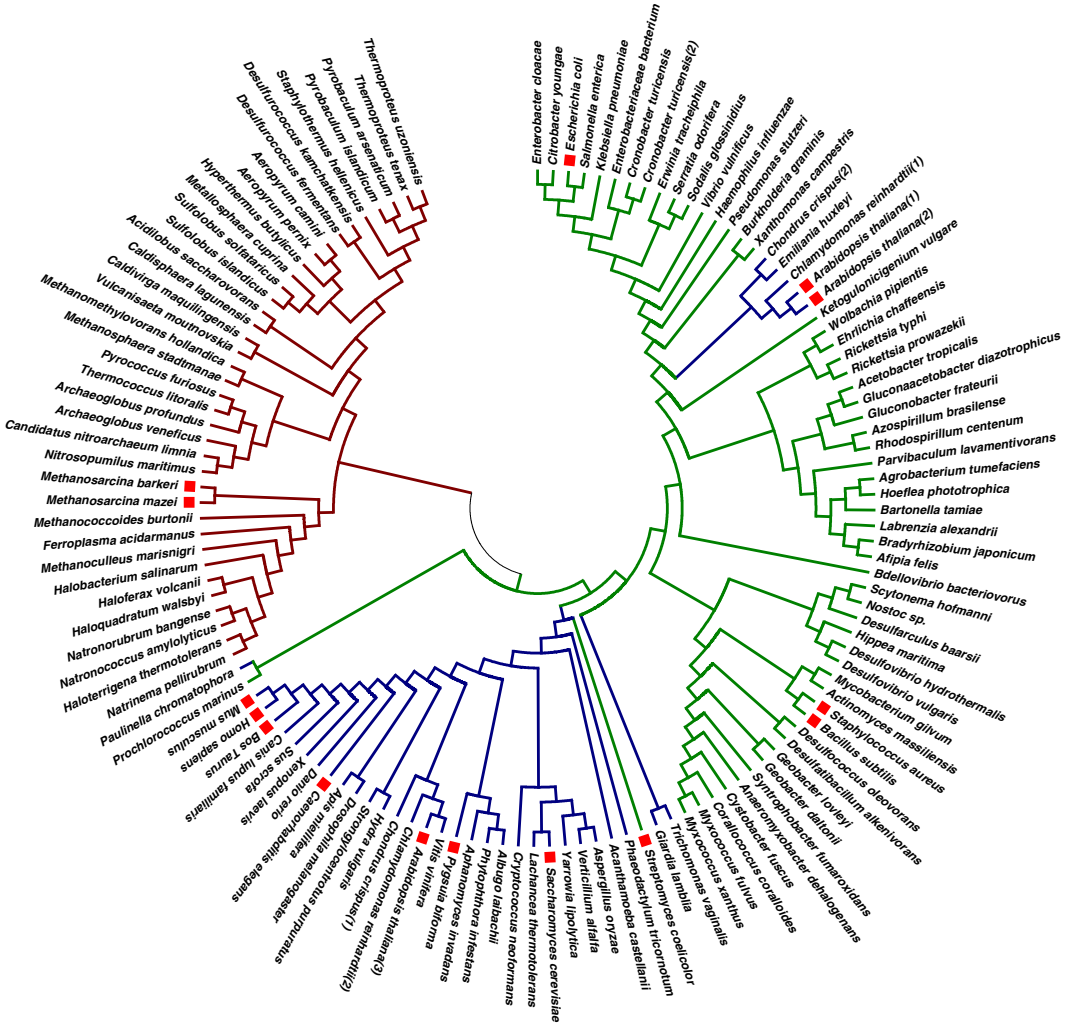

Fig. 2. Phylogenetic distribution of the CDP-DAG transferase enzymes into the three Domains. Branches are colored according to the respective domain: Brown, Archaea, Blue, Eukarya and Green, Bacteria. Eukaryote Cls (blue) are grouped with bacterial Pgs (green) sequences indicating a common evolutionary origin after Pgs diverged to Cls in most of the Eukarya subdomains. Blue sequences within the bacterial group are probably of plastidic origin (see text for explanation). On the other hand, the archeal proteins (brown) form a well-defined monophyletic group. Most of the archeal species represented here present PG in their membranes indicating that these proteins are most probably Pgs. Red squares represent the enzymes with a well-demonstrated function.

synthesized CL by a Eukaryotic-like Cls [22]. Thus, the sequence of *Streptomyces coelicolor* was expected to be grouped within the Eukarya domain. Phylogeny reported by Tian [5] showed that some α-proteobacteria also possess this enzymatic activity. In general, in [5] and this report, *S. coelicolor* sequence is basal in the Cls_T cluster, indicating that: 1) they could represent the ancestral state of eukaryote Cls (see comparison between the reconstructed ancestral Cls and *Strep. coelicolor* in supplementary figure) and 2) horizontal transfer was responsible for the spread of this protein into the few species owing these proteins and, after that, during endosymbiosis through the Eukarya domain.

3.3. Phospholipase D-like enzymes

Unlike to CDP-DAG transferases, archaeas have no proteins identified experimentally as putative Cls or Pgs with a phospholipase D activity [14]. To overcome this problem and to find the origin of the eukaryal Pgs_D, sequences of *S. cerevisiae* Pgs and *E. coli* Cls(1) were used as queries for proteins in eukaryote and bacteria. The recovered sequences in each enzyme group were used to reconstruct their ancestral states. Next, these ancestral sequences (AncCls and AncPgs) were used again as queries for searches in the three domains. Using this strategy, several archaeal proteins were recovered with significant confidence (E-value < 10^{-20}).The reconstructed tree exhibits several interesting features (Fig. 3): 1) Archaeal sequences were not monophyletic and presented a patchy distribution into several sister groups. 2) Several eukaryotic organisms presented both Cls and Pgs enzymes with a PLD-mechanism. 3) Bacterial sequences were monophyletic and were mostly restricted to the Cls_D group.

Interestingly, eukaryote Pgs was found to be related to Haloarchaea phospholipase D proteins. It is worth mentioning that several of the identified proteins were from species where the presence of CL has been demonstrated [9,10,23–26]. Since most of the Haloarchea are also represented in the CDP-DAG transferase tree, this suggests that the enzymes recovered are probably Cls that diverged to Pgs in eukaryotes. Nelson-Sathi [27,28] demonstrated that several genes with bacterial origin were horizontally transferred to a methanobacteria, which finally produced the diversification of the Euryarchaeota phylum. Moreover, they observed that the retrieved sequences marked in this work as Pgs/Cls are archaea-specific without any bacterial characteristic. Also, PLD enzymes were not included amongst the genes transferred horizontally from bacteria to methanoarchaeas [28]. Since several methanoarchaeas produce CL [25], it is tempting to conclude that the ancestral sequence was a *bona fide* Cls. This indicates that this enzyme was lost in several Euryarchaeota species and, in the case of Haloarchaea, the protein evolved to acquire a relaxed specificity, which subsequently was transmitted to the eukaryotes. Interestingly, I was unable to find any significant sequence from the rest of the archaeal

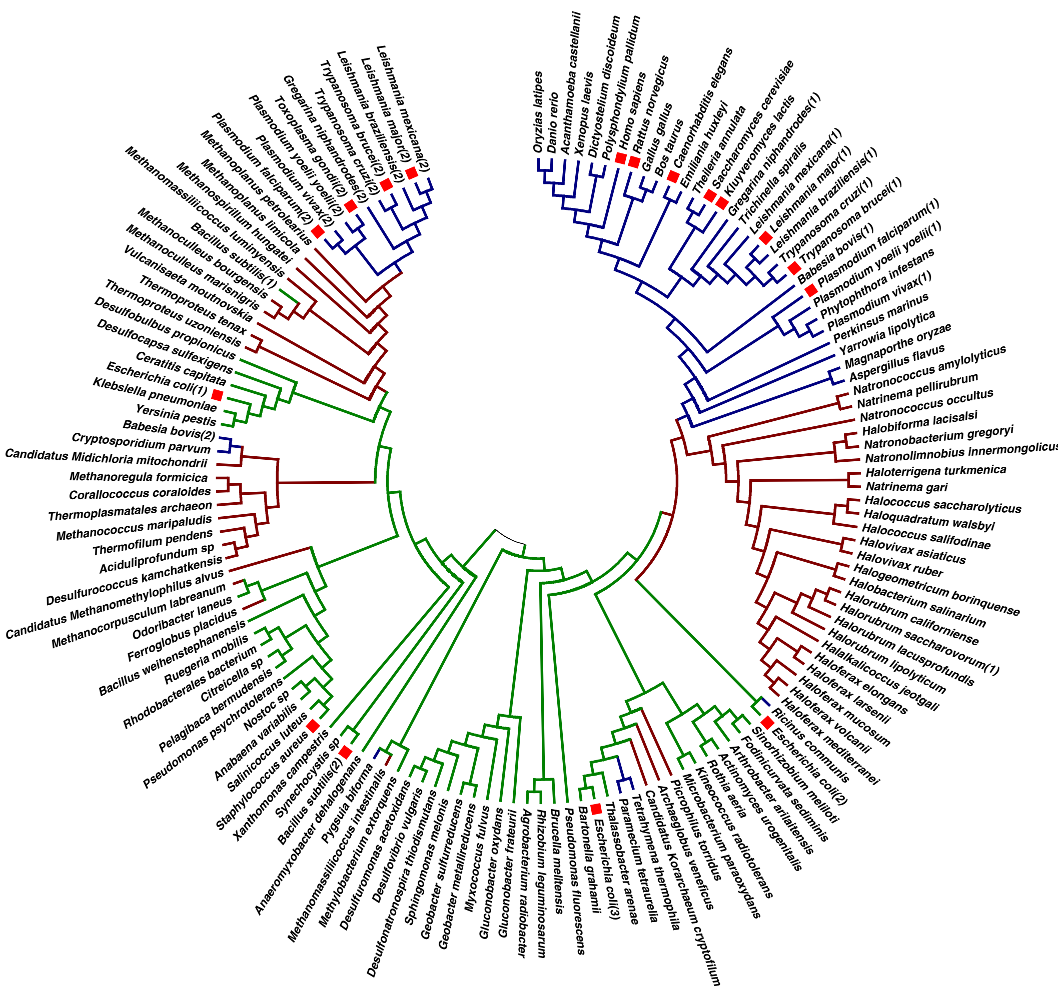

Fig. 3. Phylogenetic distribution of Cls and Pgs with a phospholipase D mechanism into the three Domains. Colors represent each domain as in Fig. 2. Topology showed a different pattern that those obtained in the CDP-DAG transferase tree. The patchy distribution is due mainly to the archaea sequences. However, it is clear that proteins from haloarchaea and eukaryotes form a monophyletic group. Some species of methanoarchaea synthesized CL, which in turn points out that ancestral proteins from those species subsequently were transferred to eukaryotes and diverged to form the modern day Pgs. Bacterial sequences (in green) are monophyletic and proteins within that group represented Cls whilst Eukarya sequences (in blue) represents Pgs. Red squares represent the enzymes with a well-demonstrated function.

phyla. It is worth mentioning that Euryarchaeota enzymes presented higher similitude with the eukaryotic Pgs than to bacteria, which might support the hypothesis that methanogens were the archaeal host in the endosymbiosis process [29].

Since CL has been almost exclusively found in Haloarchaea and some methanoarchaea, it is expected that CL evolution was coupled with the appearance of O_2-dependent oxidative phosphorylation in heterotrophic organisms. Since PLD from methanobacteria did not cluster with the haloarchaea sequences, it is rational to think that a primitive phospholipase D evolved during the transition from autotrophy/anaerobiosis to heterotrophy/aerobiosis in Euryarcheota, permitting the co-evolution of an oxygen-dependent respiratory chain with CL biosynthesis.

Kinetoplastid and Apicomplexan organism possess two proteins related to PLD. The first is included in the monophyletic group of Pgs and the second in the Cls group. As observed in Fig. 3, this last enzyme group is within the bacterial domain. This divergence from other eukaryotes, where the Cls is a CDP-DAG transferase, could be related to secondary endosymbiotic events occurring in the chromoalveolate super-group [30]. However, chromoalveolate as *Emiliania huxleyi*, *Perkinsus marinus*, *Paramecium tetraurelia* and *Tetrahymena thermophila* presented only one PLD enzyme related to the Pgs group, indicating that this enzyme was re-acquired subsequently from other endosymbiotic events.

An interesting feature is the predicted structural arrangement of the transmembrane helix in the Pgs_D and Cls_D enzymes. Bacterial sequences presented two helixes at the N-terminus of the protein. This approximately 60-aminoacid tail is apparently dispensable for the function of the enzyme and at least in *E. coli* Cls(1) this end is thought to be cleaved [31]. Several bacterial species presented several orthologous proteins to Cls(1) as in *E. coli* and *B. subtilis*. However, these last proteins presented one or no transmembrane helices [32]. It is not known if they also further process the pre-mature protein. This topology is taxonomically conserved at least in the bacterial species analyzed in this work and seems to be related with the kinetic properties of the enzyme. For example, the major Cls (Cls(1)) in *E. coli* is reversible whilst the more hydrosoluble Cls(2) not [32]. Strikingly, a third class of Cls (Cls(3)) presenting just one transmembrane helix is able to accept other phosphatidyl donor as PE for CL biosynthesis [33]. On the other hand, in haloarchaeas where CL has been documented, such as in *Haloquadratum walsbyi*, *Haloferax volcanii*, *Halobacterium salinarum* or *Natronococcus ocultus*, the identified proteins show a pattern that is quite different, presenting two or more transmembrane helices along the protein. Interestingly, the prediction for Cls from methanoarchaea shows that they possess the same topology as bacteria; the same applies for the Cls sequences from Apicomplexans and Kinetoplastids. For eukaryotes, most of the Pgs presented Cls-like topology from haloarchea. Of note, the catalytic triad characteristic of those PLD enzymes ($HXKX_6D$) are apparently located near the transmembrane sections and could be related to the specificity of the enzyme. This topology could represent a derived shared state in both domains. However, since the protein responsible for CL in archaea has not been biochemically identified, it is tempting to speculate whether this topology permits substrate recognition and/or specificity in these proteins, or, even more interestingly, the substrate used for these PLD.

3.4. A new model for CL evolution

Based on the results described above, I propose the model presented in Fig. 4. Before the endosymbiotic event, bacteria synthesized CL by the mechanism prevalent in modern day bacteria. Exceptions are the Actinomycetes. On the other hand, CL biosynthesis in Archaea would seem to be restricted to some clades where the majority synthesized PG. During the endosymbiotic event, a massive gene transfer from the bacteria to the archaea provides the basis for mixing both pathways. During the evolution of the mutualism, both pathways could be active but by chance the nowadays eukaryal pathway prevailed. This election could be dictated by bioenergetic considerations and the co-evolution of CL with the respiratory chain and the recently acquired transport mechanism, therefore, improving the energy production in the first eukaryotic common ancestor (FECA).

Primary endosymbiotic events presented new challenges for the eukaryal host. The organism emerging from such event, land plants, Rhodophytas and Chlorophytes substituted the Pgs_D with a CDP-DAG transferase, most likely the plastidic Pgs, which is also used for PG production in chloroplasts. The substitution of enzymes from the host for enzymes of plastidic origin is a well-documented process during secondary and tertiary endosymbiosis [19,20]. This could be related to the highly biosynthetic capacity of these organisms. A notable exception was observed with *E. huxleyi* (Haptophyte) which presents a Metazoan-like pathway. Afterward, a similar mechanism during secondary and, probably, tertiary endosymbiotic events within Archaeplastida gave rise to a third pathway where both enzymes were replaced by PLD-enzymes. Experimental evidence has shown the presence of CL in several members of the Alveolata (Apicomplexa) and Excavate (Kinetoplastid) groups [34,35]. In these organisms CL is present at low levels, which could be due to the presence of a pure PLD pathway. An exception was observed in *Cryptosporidium parvum*. This anaerobe Apicomplexa presents only a protein related to Cls_D. Since it did not present a CDP-transferase enzyme to supply PG, this enzyme could be involved in PG synthesis in lieu thereof.

Interestingly, two other Excavates, a Fornicata (*Giardia lamblia*) and a Parabasalia (*Trichomonas vaginalis*) presented bacterial Pgs (Pgs_T). Mostly anaerobic organisms, they did not present secondary endosymbiontic events, which supports the fact that they retained a bacterial Pgs. The presence of this protein is in line with the lipid composition of hydrogenosomes (anaerobic mitochondria) from *T. vaginalis* where PG is the only anionic phospholipid present [36]. However, little is known about the presence of anionic phospholipids in the vast majority of Chromoalveolate or Stramenopiles.

Some discrepancies between this study and that of Tin [5] concern the evolution of PLD enzymes. Here, I propose that Amoebozoa indeed presented an Opistokontha pathway in the representative *Acanthamoeba castellanii or Pygsuia biforma* [37]. However, other Amoebozoa like *Dictyostelium discoideum* and *Polysphondylium pallidum* or the Ciliates *Paramecium tetraurelia* and *T. thermophila* lost their Cls_T whilst retaining their Pgs_D. This observation indicates that the occurrence of both PLD enzymes in some species of Alveolata and Excavates is a peculiarity in these organisms more than a generality. So it is more plausible that a more realistic and parsimonious scenario includes the mixing of both archeal and bacterial enzymes giving rise to the eukaryote pathway. In this scenario, the advantages of replacing the bacterial Pgs with an analogous archeal enzyme with a PLD mechanism trigger the subsequent evolution of the Eukarya domain. Diversification in eukaryotes by divergence or serial endosymbiotic processes leads to the restructuration or the partial or complete loss of this pathway. Supporting this hypothesis is the presence of just one enzyme in eukaryotes with modified mitochondria such as hydrogenosomes (*T. vaginalis*) or mitosomes (*G. lamblia*). The loss of capacity to synthesize CL could be an ancient trait produced by the parallel loss of the canonical respiratory chain in these mitochondrial-like organelles [38].

3.5. Concluding remarks

From this brief analysis, an important question regarding CL evolution arises. What ecological advantages, if any, conferred FECA to adopt a new mechanism for CL biosynthesis? The answer might reside in the characteristic mechanism of each pathway.

First, in bacteria, CL is a secondary phospholipid present during exponential growth. Its biosynthesis is subordinated to the increase of PG during the stationary phase [39,40]. Moreover, during stressful situations like osmotic stress, CL levels increase as a compensatory mechanism to stabilize the plasma membrane [41,42]. Interestingly, a variety of PG-derivative phospholipids are produced by Cls_D in the presence

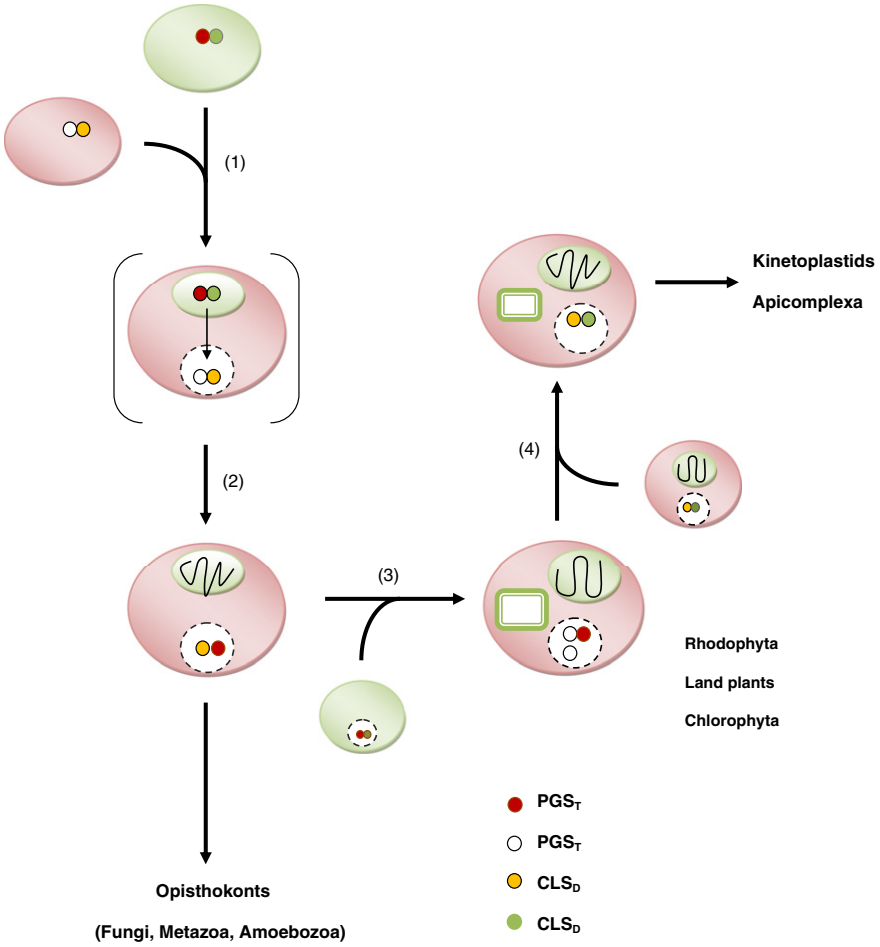

Fig. 4. Evolution of the CL biosynthetic pathway in the Eukarya domain. This model integrates the Archaea domain into the evolution of the CL biosynthesis in eukaryotes. (1) The archeal host (in pink) receipts a bacterial endosymbiont. During a transition state both pathways were possibly active. With the success of the mutualist process, the bacterial (2), by chance, the most advantageous order was positively selected and represents the modern day eukaryote pathway present in Opistokontha. This new version of the protozoa pathway suffers changes during another endosymbiotic process, which give rise to chloroplasts (3). In this case the PLD-Pgs in the eukaryote host were replaced by the plastidic Pgs. The enzyme duplicates in order to supply PG to mitochondria and chloroplast. This phenomenon is observed in land plants, Chlorophytas and Rhodophytas. No enzyme has been observed in the Glaucophyta phyla, but it is expected that they synthesize at least PG. Uptake of a red and green alga by a secondary or tertiary endosymbiosis event rearranges again the pathway. In a restricted group of Alveolates (Apicomplexa) and Excavates (Kinetoplastid), both enzymes were substituted by PLD enzymes, probably carried by the Chromoalveolate ancestor.

of several hydroxyl donors like mannitol or arabinitol [43]. In bacteria, the accumulation of PG in the membrane is the driving force for the synthesis of CL. It is worth mentioning that, since most of the enzymes with PLD domains are reversible, both hydrolysis and transphosphatydilation reactions are isoergonic; they depend on the availability of substrates for each reaction. Thus, with resumption of growth or after the stressor has been overtaken, CL is hydrolyzed back to PG by Cls [44,45]. This indicated that PG is essential for replicative growth whilst CL is for stressful situations.

Differently from bacteria, in eukaryotes mitochondria are strictly dependent on CL for the proper function of several protein engines such as oxidative phosphorylation, membrane transport proteins and mtDNA-scaffold apparatus [46,47]. Such a continuous supply of CL is only allowed if a phospholipase D-like enzyme (Pgs) is positioned at the very beginning of the pathway which also permits the direct intake of glycerol 3-phosphate produced by reduction of dihydroxyacetone phosphate (DHAP). Irreversibility of this reaction is achieved by the hydrolysis of PGP by a phosphatase poorly conserved evolutionarily. By keeping steady-state levels of PG, CL is continuously synthesized by a CDP-transferase which is a reaction driven by the hydrolysis of a high-energy bond in CDP-DAG [48]. Similarly to bacteria, CL levels rise during stress situations [63]. This response is poorly understood but might be involved inefficient ATP production by mitochondria. The continuous flux of CL in eukaryotes is also fine-tuned by CL itself by feedback-controlling the activity of Pgs [49]. Conversely, in bacteria, CL inhibits the Cls activity [50].

Evidence in *E. coli* (Cls(3)) [33,43] and *Trypanosoma brucei* (PGPS) [51] phospholipase D-enzymes demonstrates that these enzymes lack specificity, accepting any phospholipid as a phosphatidyl donor. Hence, CL or PG production could be led by the available substrate in these organisms. However, as mentioned above, in Opisthokontas, the eukaryotic pathway provides the advantage of using PG as sole substrate for Cls, avoiding the depletion of other vital phospholipids such as PE or PS. Interestingly, this pathway could be coupled to the presence of two enzymes with CDP-DAG synthase activity that is observed in Opisthokontes [52]. One isoform is dedicated to supply CDP-DAG for PS and PI biosynthesis, whilst the mitochondrial one is directed toward CL biosynthesis [53].

Interestingly, as a consequence of this new order, the morphology of mitochondrial membranes could be altered. Moreover, an increased and constant level of CL in mitochondria produced a different topology product of the non-bilayer properties of this phospholipid [54]. To support this view, recent evidence has demonstrated that liposomes containing the same composition of mitochondrial membranes are responsive to local pH gradients producing invaginations similar to those observed in the mitochondrial cristae. This phenomenon was specific for CL since other anionic phospholipids such as PS, PI or PG did not present this behavior [55–57]. Besides its non-bilayer properties

sensitive to local pH gradients, steady supply of CL acts as a fusogenic phospholipid, thus, permitting the fission and fusion of the mitochondrial reticulum [58].

CL was almost restricted to haloarchaeas [6]. This restrictive distribution triggered the diversification of this group in highly saline environments. Evolutionarily speaking, the primordial archaeal population colonizing hypersaline environments adapted this niche by overproducing anionic phospholipids. In this way, membranes with a negative surface charge in excess provide a binding site for cations through which a local concentration at the interphase is compensated with the intracellular concentration of such cations, thus, providing osmotic stability to these organisms [59]. Such a strategy followed by haloarchaeas also avoids electronic repulsion between phospholipid anionic head-groups.

Finally, CL seems to be a molecule evolutionary chosen by most aerobic cells to deal with stressful situations [23,60–62]. This is true for bacteria, eukaryotes and some archaea. CL has very particular physicochemical properties which make it suitable for this objective. Therefore, although its synthesis was modified to adjust to the energetic necessities of the Eukarya domain its intrinsic function is kept. It is tempting to conclude that most mitochondrial proteins of the inner membrane co-evoluted with the CL biosynthesis in Eukarya.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2015.03.005.

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