The membranes of the cenancestor

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Abstract The encapsulation of a genetic system was a crucial early step in the origin and early evolution of life. Genome compartmentalization is indeed required for natural selection to act upon individual entities and to maintain a certain stability of individualized genomes over generations. Compartmentalization in all contemporary cells is made possible by lipid membranes carrying out essential functions. Consequently, the most parsimonious hypothesis to explain their origin would be that the last common ancestor of living organisms (the cenancestor) already had a lipid membrane. Although all cell membranes are made up of phospholipids, which are amphiphilic molecules sharing a common general structure, there are two types of phospholipids unevenly distributed in the three domains of life. Archaeal phospholipids consist on *sn*-glycerol-1-phosphate bound to isoprenoid chains through ether links whereas Bacteria and Eucarya use *sn*-glycerol-3-phosphate bound to fatty acids via ester links. Moreover, these dissimilarities are paralleled with differences in the corresponding biosynthetic pathways. As a result, a debate has emerged between authors postulating that membranes evolved independently in the modern domains of life from a cenancestor devoid of lipid membranes and authors supporting membrane continuity between a fully cellular cenancestor and present-day lineages. This question is of major importance since it strongly influences our vision of the cenancestor nature and properties. Here, we review this controversy as well as the main arguments that have been used for and against each opposing hypothesis, and show that, in our view, recent phylogenomic results tend to confirm the presence of lipid membranes in a complex modern-like cenancestor.

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Introduction

Compartmentalization of a genetic system was a crucial, early step in the origin of life and the evolution of genomes. Not only it was probably necessary to secure a certain stability of the genetic information in primordial living entities, but it was also compulsory to individualize genomes into discrete entities that can evolve through generations (Szostak et al. 2001). With no exception, all contemporary cells are compartmentalized by membranes made up of a lipid matrix with embedded proteins in it (Singer and Nicolson 1972). These boundaries ensure the cellular integrity, separate the inside from the outside of the cell, and maintain the internal pH, osmotic conditions and the gradients of different molecules. They are responsible for selective exchanges with the environment, as they allow the diffusion of certain molecules and the active transport of others. They also take part in energy transduction because they carry both, the primary gradient-producing electron transport chains and the protein structures able to exploit the electrochemical gradient across the membranes. Finally, membranes participate in signal transduction from the environment and harbor some key enzymes that catalyze metabolic reactions such as the synthesis of the membrane lipids themselves (Berg et al. 2007).

Although it can be speculated that membranes may have had less functions in ancestral organisms with regard to modern cells, membrane boundaries are essential for life as we know it: by separating the internal medium from the environment and from other organisms, they permit self-maintenance (metabolism) and information storage that allow organisms to reproduce and undergo natural selection and, thus, Darwinian evolution. For these reasons, many authors consider that a given entity can only be considered as a living organism not only when it is self-reproducing but also when it is compartmented (Deamer 1997; Fleischaker 1990; Luisi 1997; Mavelli and Ruiz-Mirazo 2007; Moreno and Ruiz-Mirazo 2009; Morowitz et al. 1988; Oparin 1976; Pereto 2005; Szostak et al. 2001). Moreover, the chemical properties of membrane components have lead to scenarios in which the first steps of the origin of life could have been triggered by lipids and lipid-mediated compartmentalization (Chen and Szostak 2004; Deamer 1986; Deamer 1997; Hanczyc et al. 2003; Segre et al. 2001; Szathmary 2007), hence supporting a continuity between the prebiotic chemistry and the modern cells (Morowitz et al. 1991).

The outstanding chemical properties of the lipid membranes that turn them into entities of major interest in prebiotic chemistry, together with their universal distribution,

function and structure in cells from the three domains of life, strongly suggest that some kind of boundary similar to contemporary membranes may have existed in the last common ancestor of living organisms (the cenancestor). Nevertheless, the unexpected diversity of the chemical components that make up the contemporary cell membranes has raised doubts about the presence of lipid compartments in the cenancestor. Indeed, the main components of lipid membranes, the phospholipids, are made up of *sn*-glycerol-3-phosphate usually linked to fatty acid chains via ester links in bacteria and eukaryotes, whereas their archaeal counterparts use *sn*-glycerol-1-phosphate most often linked to isoprenoid chains via ether links (these differences will be detailed below). Such strong dissimilarities between contemporary membranes in archaea and bacteria/eukaryotes led some authors to propose that these lineages independently developed each type of membrane and that the cenancestor was acellular (Koga et al. 1998) or bound by mineral (iron monosulfide) bubbles (Martin and Russell 2003), whereas others invoke a greater continuity between the cenancestor and the subsequent three domains of life by proposing that the cenancestor bore mixed membranes with properties that can be inferred from modern organisms (Peretó et al. 2004; Wächtershäuser 2003).

The presence or absence of a membrane in the cenancestor, its chemical nature and the early evolutionary process which originated the subsequent domains of life are major issues when trying to infer the characteristics of the cenancestor and to determine the functions that it could have been able to carry out. Addressing these questions requires a thorough revision of current knowledge on lipid diversity and its impact on the different suggested scenarios. In this chapter, we will first describe some features common to all contemporary membranes and, afterwards, the differences that have been put forward as contradictory with the presence of lipid membranes in the cenancestor and the scenarios that have been proposed to take into consideration these observations. We will revisit the apparent dissimilarities that have been observed in biological membranes and highlight a cellular hypothesis that we think to be the most plausible based on current data.

Universal characteristics of biological membranes

Together with membrane proteins, the main components of modern membranes are phospholipids (although other types of lipids are also of major importance). Phospholipids are amphiphilic molecules, i.e. they bear in the same molecule hydrophilic regions interacting with water and hydrophobic regions segregating from the aqueous medium. The hydrophilic

part is called the head, and the hydrophobic parts are the tails (Fig. 1). Phospholipids are built around a glycerol backbone linked to a phosphate group either in its first or its third carbon, what raises two different enantiomers: *sn*-glycerol-1-phosphate (G1P) and *sn*-glycerol-3-phosphate (G3P), respectively. Several different polar groups can be linked to the phosphate residue: serine, *myo*-inositol, ethanolamine, glycerol, etc. The phosphate and the polar group form the hydrophilic head of the phospholipid. The remaining two free carbons of glycerol are usually linked to long carbon chains either by ether or ester bonds, forming the characteristic hydrophobic tails. These carbon chains are very diverse, but they mostly belong to two main families: fatty acids synthesized from the condensation of acetyl-CoA units, and isoprenoid chains synthesized from universal activated forms of isoprene that serve as precursors (isopentenyl pyrophosphate, IPP, and dimethylallyl diphosphate, DMAPP).

The currently accepted fluid mosaic model of membranes was born from the attempt to minimize the thermodynamic requirements of phospholipids and membrane proteins or, more precisely, maximize the hydrophobic interactions in the one hand and the hydrophilic interactions in the other hand (Singer 2004; Singer and Nicolson 1972). Phospholipids form bilayers in which the hydrophilic heads interact among them and with water in both sides of the membrane, while the hydrophobic tails face each other in the central part of it and, thus, maximize the hydrophobic links away from water. Essential integral proteins are inserted into this bilayer: the hydrophobic parts of proteins interact with the hydrophobic central part of the phospholipid matrix whereas the polar regions of the proteins are excluded from the membrane to one or both sides of it.

The term “mosaic” in this model refers to the mixture of phospholipids and proteins within the plane of the membrane, contrary to competitor models (Benson 1966; Davson and Danielli 1952; Robertson 1964). The model is also “fluid” since the membrane proteins and the phospholipids can diffuse in it, allowing random movements but also forming specific aggregates when required. Importantly, phospholipids can freely move within their half-bilayer, although the transfer from one side to the other is thermodynamically unlikely and must be mediated by specific integral proteins called flippases (Daleke and Lyles 2000). In the same way, integral proteins can move within the plane of the membrane but most often cannot change from one polarization to the other because of thermodynamic constraints. This is relevant to another major feature of contemporary biological membranes: they are asymmetric, that is the composition of each half-bilayer (inner/outer) is different from the

other. The most common example is the asymmetric distribution of oligosaccharides, which are specifically found in the outside of the cell linked to lipids and proteins in so-called glycolipids and glycoproteins, and absent from the inner half-bilayer. Other examples are the specific polarities of integral proteins and the presence of peripheral proteins bound only in one side of the membrane.

Although here we have focused our attention on phospholipids and proteins, other components take part in the membrane composition, as for example many isoprenoid derivatives, which bear essential functions both for the cell (pigments) and membrane (fluidity control by sterols) functioning. However, as the truly minimal components of contemporary membranes are phospholipids, most of our discussion will concern their characteristics and evolution.

Two different phospholipid types in the three domains of life

Despite all phospholipids share the aforementioned common structure that confers them their chemical and thermodynamic properties, major differences have been observed between the phospholipids occurring in archaea and those from bacteria and eukaryotes (Fig. 1). The most important one, which is also the only one without any known exception, is the utilization of opposite glycerol phosphate enantiomers: archaea use G1P whereas bacteria and eukaryotes use G3P in their phospholipids. This is correlated with their enzymatic stereospecificities, namely the presence in archaea of a *sn*-glycerol-1-phosphate dehydrogenase (G1PDH) only able to produce G1P, while bacteria and eukaryotes have a *sn*-glycerol-3-phosphate dehydrogenase (G3PDH) synthesizing the opposite enantiomer (Kito and Pizer 1969; Koga et al. 1998; Nishihara and Koga 1997). G1PDH and G3PDH are non-homologous enzymes that belong to totally unrelated protein families (Daiyasu et al. 2002; Koga et al. 1998). This raises the question of which enzyme, if any, was present in the cenancestor.

Moreover, archaea are known to bear isoprenoid chains in their phospholipids and have been thought to completely lack fatty acids until very recently (De Rosa et al. 1986; Kates 1992), whereas bacterial and eukaryotic phospholipids usually incorporate fatty acids instead of isoprenoid chains. These differences are paralleled with dissimilarities in the related biosynthetic pathways. On the one hand, the fatty acid biosynthesis pathway was unknown in archaea; on the other hand, isoprenoid precursors, IPP and DMAPP, although universally

distributed, are synthesized by two independent non-homologous pathways that were thought to be exclusively related to some groups of organisms: the mevalonate pathway (MVA) in archaea and eukaryotes and the methylerythritol phosphate pathway (MEP) in bacteria and plastid-bearing eukaryotes (Lange et al. 2000). All these contradictions among the three domains of life appeared to belie the presence of hydrophobic tails in the hypothetical phospholipids of the cenancestor.

Finally, in the archaeal phospholipid archetype, isoprenoid chains are bound to G1P via ether links, whereas in bacteria and eukaryotes fatty acids are usually bound to G3P through ester links (Fig. 1). However, many exceptions to this splitting are known in modern organisms: ether-linked lipids have been detected in proportions up to 25% in animal membranes (Mangold and Paltauf 1983) as well as in at least six different bacterial phyla, including Firmicutes, Proteobacteria, Planctomycetes, Thermotogae, Aquificae and Thermodesulfobacteria (Damste et al. 2007; Huber et al. 1992; Jung et al. 1994; Langworthy et al. 1983; Rutters et al. 2001; Sinninghe Damste et al. 2002; Weijers et al. 2006).

First uncertainties: was the cenancestor compartmented? The acellular hypothesis

First of all, it must be noted that most of the hypotheses proposed only discuss the descent of the archaeal and the bacterial lineages from the cenancestor, the eukaryotes being assumed to have evolved subsequently from these primordial lineages. In fact, many hypotheses consider that eukaryotes originated from a symbiosis event between an archaeal and a bacterial organism (e.g., Martin and Muller 1998; Moreira and Lopez-Garcia 1998). This is based on the apparent chimerical nature of eukaryotic genomes, which show a mix of genes of putative archaeal origin (usually the informational genes) and genes of putative bacterial origin (most operational or metabolic genes). Independently of the debate about the nature and origin of the first eukaryotes (Gribaldo et al. 2010), discussions about ancestral membranes traditionally either accommodate only chimeric hypotheses or simply ignore the problem of the evolution of the eukaryotic membranes.

Until the late 1990's, the cellular nature of the cenancestor was not a matter of hot debate, contrarily to what it has become since then. The fact that all organisms were limited by lipid membranes made up of phospholipid and proteins and respecting the fluid mosaic model was simply regarded as a consequence of the vertical inheritance of these

characteristics from early ancestors. Nevertheless, the different composition in archaeal phospholipids with respect to their bacterial counterparts was already known (see above). Of these dissimilarities, the only one for which any exception had been detected was the stereochemistry of the glycerol phosphate backbone (G1P in archaeal *versus* G3P in bacteria and eukaryotes).

In this context, the isolation and sequencing of the archaeal G1PDH gene (Koga et al. 1998; Nishihara and Koga 1997), followed by the discovery that G1PDH and G3PDH were non-homologous, led to the idea that the two enantiomer biosynthesis pathways had independent origins. On this basis, Koga *et al.* (1998) proposed that each glycerol phosphate dehydrogenase (and its related phospholipid type) had emerged independently in the ancestors of Archaea and Bacteria, triggering the differentiation of these two domains of life from a phospholipid-lacking cenancestor. This hypothesis entails that the first compartmented organisms were the respective ancestors of Bacteria and Archaea and, thus, that the cenancestor was acellular (Koga et al. 1998). In order to describe the nature of the cenancestor, these authors invoked previous suggestions that primitive metabolism could have emerged and concentrated in a chemical manner on pyrite (FeS_2) surfaces (Wächtershäuser 1988). Accordingly, they proposed that soluble metabolites, some enzymes and even the genetic machineries originated in this type of non-compartmented system. At this chemical stage, a racemic mixture of G1P and G3P could have accumulated, providing phospholipid precursors prior to the independent emergence of stereospecific catalysts and the subsequent cellularization of the bacterial and archaeal lineages. Eukaryotes would lately have evolved from an endosymbiosis event between the two primordial domains of life (Fig. 2A).

The most interesting point of this hypothesis is that it straight addresses the question of the presence and the nature of a membrane in the cenancestor, which became a debated open matter since then. Nevertheless, this scenario raises several major issues: (i) although pyrite surfaces could be interesting concentrating hypotheses for the prebiotic chemistry that could have provided the building blocks involved in the first steps of life, the cenancestor was an organism that certainly had transcription, translation (included the genetic code) and some protein enzymes (Delaye et al. 2005; Gogarten et al. 1989; Koonin 2003; Kyrpides et al. 1999; Ouzounis et al. 2006). Therefore, it is very difficult to conceive that such complexity may have occurred in free solution without the diffusion of the intermediates (de Duve and Miller 1991; Oparin 1976); (ii) reproduction of genetic elements must be linked to the entities that confer them a reproductive advantage to allow Darwinian evolution; therefore, spatial

confinement is indispensable (de Duve 2005; Oparin 1976); (iii) amphiphilic molecules have been obtained under plausible prebiotic conditions (Apel and Deamer 2005; McCollom et al. 1999), and amphiphilic compounds able to spontaneously form membranous vesicles under appropriate conditions have been detected in meteorites (Deamer 1986; Deamer and Pashley 1989), so the prebiotic synthesis of lipid molecules and membranes does not seem to be a restricting step that should absolutely be dependent on biosynthesis by biological catalysts (Deamer et al. 2002).

To summarize, the acellular scenario could be seen at first sight as the simplest response to the puzzling observation that two very different phospholipid types exist in the domains of life, which are correlated to non-homologous biosynthesis pathways. Nevertheless, this no-compartment hypothesis raises more problems than it gives answers, since it requires the emergence of an extraordinary complexity from very simple chemical mechanisms without providing neither a satisfying concentration mechanism nor a plausible driving force. Moreover, this idea is contradictory in that it proposes an ancestral system that could have been able to reach complex autoreplication and complex metabolism but not to produce amphiphilic lipid molecules that, paradoxically, can be easily synthesized in prebiotic conditions.

Did the cenancestor have boundaries different from lipid membranes? The inorganic compartments hypothesis

As we have just seen, the apparent irreconcilable differences between archaeal and bacterial/eukaryotic phospholipids first led to the proposal of an acellular cenancestor. However, non-compartmented systems can hardly account for the probable complexity attained by the cenancestor and are not able to put together the genetic elements (genotype) and metabolic functions (phenotype) essential to apply Darwinian selection and evolution (de Duve 2005; Tawfik and Griffiths 1998). Since compartments were required in the cenancestor but lipid membranes seemed impossible to some authors, the obvious third possibility was that the cenancestor was compartmented in some other kind of structure. As pioneers of this idea, Oparin and coworkers (1976) tried to prepare spherical aggregates (coacervates) from macromolecular components such as Arabic gum, gelatin and histones, but these structures were unsuitable to model primordial cells because their synthesis in prebiotic conditions is very unlikely and their thermodynamic stability is low (Monnard and Deamer 2002; Walde et

al. 1994). Alternatively, hydrothermal vents had been proposed as likely sites of prebiotic synthesis of organic molecules since they provide a concentrating mechanism (physical limits to slow down free diffusion in the ocean), Fe-S and Fe-Ni-S centers that may participate in primordial catalysts, and a continuous redox energy source to allow the synthesis of possible biochemical building blocks (Russell 1997). Moreover, in these sites, inorganic 'bubbles' are formed by the spontaneous precipitation of iron monosulfide. This led some authors to speculate that not only some prebiotic chemistry but all the evolutionary process from the first prebiotic chemistry up to the cenancestor had taken place in a submarine hydrothermal vent, and that the respective ancestors of archaea and bacteria cellularized and escaped the original vent only after two independent lipid inventions in a very late period of this process (Fig. 2B, Koonin and Martin 2005; Martin and Russell 2003).

In this scenario, the transitions between the RNA, RNA-protein and DNA worlds, as well as the development of transcription and translation, all took place in these hydrothermal compartments. The cenancestor itself would have been part of this hydrothermal scenario: it would correspond to a network of iron monosulfide compartments containing retrovirus-like RNA chromosomes (the cenancestor is here thought to be incapable of DNA replication, since the replication machineries are not orthologous in archaea and bacteria (Koonin et al 2006)). These genetic elements were the agents of variation (mutation) and natural selection for self-replication. Within the mineral boundaries, they would entail the emergence of intricate autocatalytic networks able to make DNA, RNA, and proteins, and to sustain a core and an intermediate enzymatic metabolism (nitrogen metabolism, amino acid, nucleic acid and cofactor biosyntheses). As the hydrothermal vent grew through the formation of new iron monosulfide cavities, the most successful genetic elements (the fastest replicators) would have diffused into them, allowing the propagation of such ensembles within the spreading mineral compartment network. In this context, two distinct systems for phospholipid biosynthesis might have evolved and coupled with the emerging DNA genomes. Once membranes, cell walls, DNA genomes and bioenergetic pathways came into place, they independently allowed the emergence of the first free-living organisms, namely the respective ancestors of bacteria and archaea. As in the previous scenario, eukaryotes would have emerged from the symbiosis between an archaeon and a bacterium (Martin and Russell 2003).

This hypothesis is of major interest, especially in the early prebiotic era, as a mechanism of production and concentration of biochemical building blocks. Nevertheless, it encounters major problems when it is applied to subsequent evolutionary stages, in particular

the one corresponding to the cenancestor. First of all, in contrast with claims formulated by the authors of this hypothesis (Koonin and Martin 2005), this hypothesis does not allow Darwinian evolution since the replication of the autocatalytic units is not necessarily coupled with the formation of new compartments. In other words, in this hypothesis the most efficient autoreplicative units would be able to control their own replication and would freely diffuse into the new formed 'bubbles', but, as they would have no control at all on the formation of those bubbles, there would be no coupling between genetic material replication and organismal multiplication. Therefore, those compartments could neither be considered as autopoietic entities (Fleischaker 1990) nor as individuals undergoing natural selection (de Duve 2005). Another problem related to this scenario concerns the emergence of lipid membranes: the biosynthesis pathways producing the lipid components that would integrate the future archaeal and bacterial membranes are proposed to have evolved and colonized the inorganic components in the same way as the DNA genomes and other elements. However, this hypothesis fails to propose a realistic function for those molecules, and, thus, a selective advantage to explain the success of their biosynthetic pathways. It could be naturally assumed that the presence of membranes allowed the encapsulation of genetic or metabolic units. But, if these elements were already encapsulated in lipid membranes, the iron monosulfide compartments at the center of this hypothesis would become unnecessary.

There are other arguments strongly related to the problem of lipid membranes that weaken Martin and Russell's hypothesisthis model: as we will discuss later, several membrane proteins (e.g., ATPases and the membrane-targeting SRP system) most likely existed in the cenancestor (Gogarten et al. 1989; Gribaldo and Cammarano 1998; Mulkidjanian et al. 2007), what raises the question of their location and function in the cenancestor if it was bounded by inorganic compartments (Jekely 2006; Pereto et al. 2004). To answer to this concern, the authors of this scenario refined it by incorporating the idea that some kind of aliphatic chains could have been produced in the hydrothermal conditions that allowed the formation of hydrophobic patches in which the ATPases and the SRP systems could be embedded (Koonin and Martin 2005). But, again, if lipid vesicles able to bear ATPases and the SRP system existed, why these lipid components did not encapsulate also the genetic entities? And even if the genetic entities were external to the vesicles, what was the driving force to evolve membrane-targeting systems (the SRP complexes) if the genetic elements encoding these proteins were not linked to them? Only two ways to link together a lipid vesicle and some genetic elements can be proposed: either to covalently fix them to each

other (and there is no evidence for this) or that the genetic elements be encapsulated by the lipid layers, as in modern cells. Finally, it has been shown that other metal sulfides different from iron monosulfides would do better in providing the energy for the synthesis of biomolecules, questioning the likely importance of FeS cavities in prebiotic steps (Mulkidjanian 2009; Mulkidjanian and Galperin 2009). However, regardless to the nature of the metal involved in the construction of such a mineral boundary, the criticisms about the presence of mineral boundaries in the cenancestor remain.

To conclude, the hypothesis based on hydrothermal mineral compartments may provide an interesting scenario for prebiotic chemistry, but it seems implausible for subsequent evolutionary stages, including the cenancestor. Indeed, since the proposed inorganic boundaries were not linked to the autoreplicative entities, this hypothesis falls into the same problems as the acellular hypothesis: a boundary able to link together all genomic elements is necessary to explain what we know about the cenancestor. Moreover, it is noteworthy that the inorganic boundaries proposal needs to assume some kind of lipid structures prior to the divergence of the prokaryotic lineages. A more parsimonious hypothesis would be that the cenancestor already bore a lipid membrane.

Was the cenancestor bounded by heterochiral membranes? The pre-cell theory

As we have seen, trying to explain the dissimilarities between the archaeal and the bacterial/eukaryotic phospholipids first led to hypotheses assuming the lack of lipid membranes in the cenancestor. However, other authors addressed the possibility that both types of phospholipids actually existed in the cenancestor and that the contemporary differences in archaeal and bacterial cells are secondary and related to the divergence of these two lineages from the last common ancestor.

This hypothesis was first postulated as an extension to Kandler's pre-cell theory. Kandler's hypothesis (1994) depicts the cenancestor not as an organism but as a population of self-reproducing entities endowed with metabolism and genetic information, and unable to limit frequent mutual exchange of genes. In spite of the frequent gene exchange, not all cells would have been identical within the population. These entities are called pre-cells because of their incapacity to keep their genetic information apart from the rest of the community, but from a membrane point of view, these entities limited by lipid membranes can be considered as perfectly cellular. Kandler assumes that the bacterial lineage diverged first from this

community, which continued to evolve in its own way. A second lineage of organisms would have diverged later to become the Archaea, then a third lineage started a symbiotic relationship with a bacterium to originate the Eucarya (Kandler 1994).

Based on this scenario of a 'cenancestral population', Wächtershäuser addressed the question of the chemical nature of the boundaries of the pre-cells (Wächtershäuser 2003). First, he assumed that hypothetical hybrid heterochiral membranes (i.e., with a mix of G1P and G3P) may have existed although they would have been less stable than homochiral ones. The pre-cells are proposed to have borne such heterochiral membranes, the components of which could have been synthesized in a racemic way either by inorganic catalysts or by primitive non-stereospecific enzymes (Fig. 2C). In these pre-cells, spontaneous lipid segregation due to physico-chemical stability differences between the two phospholipid types would have occurred. Fusions and fissions among cells are proposed to explain the mutual genetic exchange and also to explain how the heterochiral membranes progressively segregated into two opposite homochiral subpopulations. The emergence of one G3PDH stereospecific enzyme would have definitely fixed one kind of homochiral membrane in the bacterial lineage, whereas the subsequent advent of the G1PDH stereospecific enzyme would have set the opposite homochiral membranes in archaeal ancestors. Shifts from one chirality to the other would have been prevented by the lower stability of the heterochiral intermediates (Wächtershäuser 2003). This hypothesis of lipid segregation based on chirality has been recently extended to the hydrophobic tails: whereas G1P-fatty acids and G3P-fatty acids are enantiomers, phospholipids made up of G1P-isoprenoids and G3P-isoprenoids are diastereomers (non-enantiomeric stereoisomers with at least two chiral centers). Since diastereoisomers have different physicochemical characteristics that enantiomers have not, different combinations of these components are not supposed to segregate in the same way. Consequently, the hydrocarbon chains are proposed to play a supplementary role in the segregation of heterochiral membranes in addition to the chirality of the glycerol phosphate backbones (Koga 2011).

This scenario takes into account both the existence of ancestral compartmented organisms and the characteristics of modern membranes. Nevertheless, it does not provide an explanation for the synthesis of phospholipid components, keeping the door open to either an inorganic biosynthesis or an enzymatic non-stereospecific pathway. The possibility of inorganic biosynthesis, such as the anabolism on colloidal surfaces or microcrystalline particles with metal-sulfide structures proposed by Wächtershäuser, closely brings together

the origin of life and the cenancestor time, and addresses the question of the capabilities of the cenancestor and its independence with respect to its environment. The possibility of the enzymatic non-stereospecific synthesis of phospholipids in the cenancestor has been criticized by arguing that it would make the cenancestor the most versatile organism that never existed (Koonin and Martin 2005). Nevertheless, this argument is weakened if we envisage that the putative enantiomeric lipid mix in the cenancestor was the result of a limited number of non-stereospecific enzymes. When Wächtershäuser hypothesis was first postulated, there was no available information concerning the early evolutionary history of the phospholipid biosynthesis pathways. We will treat this aspect later (see below) to show that actually the majority of the enzymatic machinery responsible for phospholipid biosynthesis in modern cells can most likely be traced back to the cenancestor.

In addition to the biotic or abiotic nature of the source of phospholipids, there is another important concern about this hypothesis. The whole hypothesis depends on the assumption that heterochiral membranes are less stable than their homochiral counterparts. At first sight, this looks plausible since, for example, racemic mixtures of D- and L-myristoylalanine undergo rapid segregation (Nassoy et al. 1995) and mixed bacterial-archaeal liposomes appear to be less stable than uniform ones (Longo et al. 2007). However, the opposite observation (a higher stability of hybrid eukaryotic/archaeal liposomes) has also been reported in other independent studies (Fan et al. 1995). To solve this ambiguity, new experiments have recently been carried out, showing that hybrid liposomes are as stable as homochiral liposomes and the stability is much more correlated to the hydrocarbon chain length than to the backbone chirality or chain nature (Shimada and Yamagishi 2011). These results, which explain differences among previous works, are at odds with the theoretical premise of Wächtershäuser's pre-cell scenario.

A phylogenomic exploration of the enzymatic capacities of the cenancestor to synthesize phospholipid backbones

In the previous sections, we have discussed the differences between the two archetypical phospholipids and the hypotheses that have been proposed to take them into account when inferring the nature of the cenancestor membranes. The last hypothesis discussed above bumped into a lack of information concerning the enzymatic capacities of the cenancestor, preventing the presentation of a plausible way to synthesize its membranes. In the following,

we will explore the evolutionary history of the enzymes involved in this process in contemporary cells.

As already mentioned, the most distinctive feature between the archaeal and the bacterial/eukaryotic phospholipids is the glycerol phosphate stereochemistry (G1P and G3P, respectively). This is the only characteristic without any known exception so far. G3P is a very common metabolite that contemporary cells synthesize from multiple substrates, such as dihydroxyacetone phosphate by a flavin-dependent G3P-dehydrogenase (*glp*), or glycerol by a glycerol kinase (*glpK*). If we solely consider the presence of glycerol phosphate in cells, G3P can actually be detected *in vivo* in heterotrophic archaeal cells incubated with glycerol (Nishihara et al. 1999). Moreover, genes similar to G3P-producing enzymes have been detected not only in bacteria and eukaryotes, but also in archaea (Peretó et al. 2004). These arguments suggest that G3P is a universal metabolite but, as archaea do not use it for their phospholipids, this cannot be accepted as enough evidence to infer that G3P participated in cenancestral phospholipids. As a result, much interest has focused on glycerol phosphate dehydrogenases responsible for G1P/G3P supply specifically related to phospholipid biosynthesis in modern cells. Primary sequence comparisons and tertiary structure analyses show that archaeal G1PDH and bacterial/eukaryotic G3PDH derive from different ancestral enzymes, despite they share the same substrate and coenzyme requirements (Daiyasu et al. 2002; Koga et al. 1998). Nevertheless, the evolutionary history of both enzymes is very complex since each one belongs to different large enzyme superfamilies.

G1PDH is part of a superfamily that also contains glycerol dehydrogenase (GDH), alcohol dehydrogenase (ADH) and 3-dehydroquinate synthase (DHQS). All of them share a great functional and structural resemblance, including the oxidoreduction of NAD^+ (Carpenter 1998, Ruzheinikov 2001). A phylogeny of this superfamily shows that horizontal gene transfers (HGT) do exist but are limited in number and do not mask the phylogenetic origin of each sub-family: GDH is widespread among bacteria, ADH and DHGS are characteristic of bacteria and eukaryotes and G1PDH of archaea. Thus, although neither function seems to be universal, the ancestral existence of members from this superfamily in the three domains of life is supported, so that the presence of at least one primitive NAD^+-dependent dehydrogenase of this superfamily can be inferred for the cenancestor. Since contemporary enzymes from the three domains of life are not congruent with regard to their substrate specificities, it can be reasonably proposed that this ancestral enzyme had a nonspecific

dehydrogenase activity from which modern characteristics rose, and G1P synthesis cannot be excluded (nor definitely confirmed) in the cenancestor (Peretó et al. 2004).

Very similar arguments can be provided for G3PDH, which is distantly related to two other enzymes, UDP-glucose 6-dehydrogenase (UDPGDH) and 3-hydroxyacyl-CoA dehydrogenase (HACDH) (Kavanagh et al. 2003). All these enzymes also share a NAD^+-dependent oxidoreduction mechanism. Phylogenetic analyses of this superfamily show three groups of sequences, each of them corresponding to one of the three functions previously cited (Peretó et al. 2004). Some HGTs can be detected, but they do not hide the origin of each sub-family gene. UDPGDH is a universally distributed subfamily involved in synthesis of different polymers in eukaryotes and bacteria (Arrecubieta et al. 1994; Binari et al. 1997; Dalessandro and Northcote 1977). In addition, the presence of D-glucuronic acid in some archaeal cell walls (Kandler 1994) suggests that these enzymes could be involved in its biosynthesis. This implies major metabolic roles of this subfamily in the three domains of life. Similarly, HACDH is widely distributed in the three domains of life and is involved in different polypeptidic enzymes carrying out many diverse functions (Hiltunen and Qin 2000; Pereto et al. 2004; Youngleson et al. 1989). Thus, among members of the G3PDH/UDPGDH/HACDH superfamily, only G3PDH is limited to bacteria and eukaryotes (the rare homologous genes found in archaeal genomes have probably been acquired by HGT). As the two other subfamilies are universally distributed, the whole superfamily is likely to have had at least two representative genes in the cenancestor (maybe more), despite their function cannot precisely be established. Consequently, it cannot be excluded that at least one of these ancestral promiscuous enzymes was capable of synthesis of G3P among other functions, before functional divergence happened. In that case, G3PDH function could have been lost in the archaeal lineage just because catabolic G3P formation could be catalyzed by other enzymes (glycerol kinase and flavin-dependent G3PDH, as previously seen) and specific-G3P synthesis for phospholipid biosynthesis became unnecessary in this lineage (Peretó et al. 2004).

In summary, although G1PDH and G3PDH belong to two separate superfamilies, ancestral representatives from both were most likely present in the cenancestor, where they could have provided G1P and G3P non-specifically. Thus, the cenancestor would have been endowed with hybrid, heterochiral membranes (Fig. 2D).

Could the cenancestor synthesize hydrocarbon lateral chains?

Apart from the backbone chirality, another major difference between archaeal and bacterial/eukaryotic phospholipids is the utilization of isoprenoids as hydrophobic chains in the former whereas fatty acids are used in the latter. This is stressed by the observation that, although isoprenoids exist in all organisms and their precursors (IPP and DMAPP) are the same in the three domains of life, two different non-homologous isoprenoid biosynthesis pathways are unevenly distributed in the three domains of life: the mevalonate pathway (MVA) is typical of archaea and eukaryotes, whereas the methylerythritol phosphate pathway (MEP) is characteristic of bacteria and plastid-bearing eukaryotes. This disparity is surprising if we take into account that isoprenoids are present in all organisms as essential components of phospholipids, electron transport chains, pigments or hormones (Lange et al. 2000; McGarvey and Croteau 1995).

Recent work has tried to reconcile the ubiquitous utilization of isoprenoids with the apparent lack of an ancestral way to synthesize their precursors. First, the MEP pathway has been confirmed to be restricted to bacteria and plastid-bearing eukaryotes (Matsuzaki et al. 2008). This pathway is widely distributed in bacterial genomes, supporting that it was present in the last common ancestor of bacteria. Plastids are known to have evolved from endosymbiotic cyanobacteria and transmitted major metabolic routes to their eukaryotic hosts (Martin et al. 1998). Although some phylogenies of the MEP pathway genes do not support the expected cyanobacterial origin, all of them support a relatively recent bacterial origin of these eukaryotic enzymes (Brinkman et al. 2002; Matsuzaki et al. 2008; Moustafa et al. 2008). This limited taxonomic distribution makes the presence of the MEP pathway in the cenancestor unlikely.

Therefore, the search of plausible routes to produce isoprenoids in the cenancestor has focused in the alternative MVA pathway. Although it was supposed to be limited to eukaryotes and archaea, some bacterial representatives have been characterized and shown to participate in isoprenoid precursor synthesis among other functions (Voynova et al. 2004; Wilding et al. 2000). These bacterial genes were first thought to be the result of HGTs from the two other domains (Boucher and Doolittle 2000; Wilding et al. 2000). Surprisingly, when similarity searches were carried out in bacterial genomes, a much more wide diversity of bacteria bearing enzymes of the MVA pathway was identified (representatives are found in 6 different phyla). Phylogenetic analyses of each enzyme of the pathway showed that these

genes did not branch among the sequences from the two other domains of life as it would be expected for HGTs, but systematically clustered together in clades separated from eukaryotic and archaeal sequences. Moreover, although some cases of HGT among bacterial species were identified, bacterial sequences in these phylogenies most often respected the monophyly of main bacterial taxa, supporting vertical inheritance as the major mechanism to explain the presence of the pathway in these bacteria. Finally, biochemical properties only shared among bacteria and absent from the two other domains of life have been pointed out (Bochar et al. 1999; Friesen and Rodwell 2004; Hedl et al. 2004; Scher and Rodwell 1989), supporting that these characteristic are enzymatic synapomorphies that evolved in the bacterial lineage after the divergence of the three domains of life (Lombard and Moreira 2011b).

This result was of major relevance in resolving the apparent opposition between the universal distribution of isoprenoid precursors and the so far uneven distribution of their biosynthetic pathways: although the MEP pathway was really limited to bacteria and plastid-bearing eukaryotes, supporting its bacterial origin, the mevalonate pathway was shown to be ancestral to each domain of life, suggesting a common inheritance from the cenancestor and subsequent modifications to explain specificities in each domain of life. Many bacteria would have lost the mevalonate pathway in favor of the functionally redundant MEP pathway, as well as archaea are known to have changed the last steps of the MVA pathway with regard to the typical eukaryotic/bacterial counterpart (Lombard and Moreira 2011b).

The assumption that fatty acids are missing in archaea (De Rosa et al. 1986; Kates 1992), where isoprenoids are the main hydrophobic chains in phospholipids, represented a similar difficulty than the uneven distribution of the isoprenoid precursor biosynthesis pathways. Though, significant amounts of the total phospholipid side chains (from 11.3 to 89%) were recently determined to contain fatty acids in five different euryarchaeotal classes (Gattinger et al. 2002). Furthermore, genes involved in fatty acid biosynthesis were described as the largest functional gene family in the crenarchaeote *Sulfolobus solfataricus* (She et al. 2001). These arguments suggest that not only fatty acids can be found in archaea, but also that they are widespread in this domain of life and can occasionally be major components of archaeal membranes. In agreement with this, sequence similarity searches carried out to identify archaeal homologues of fatty acid biosynthesis and degradation genes found that all genes, except those of the acyl-carrier protein machinery (ACP), have more or less distantly related homologues in a wide diversity of archaeal genomes. Genes involved in the fatty acid biosynthesis pathway have very complex evolutionary histories including many duplication

and fusion events with other enzymatic units, what makes the early evolution of these families very difficult to study. Nevertheless, recent phylogenomic analyses showed that the presence of the first step of the fatty acid biosynthesis (acetyl-CoA carboxylation) in the last common ancestor of archaea can not be excluded (Lombard and Moreira 2011a).

In summary, the observation that significant amounts of fatty acids exist in membranes from a wide diversity of archaea, the finding of fatty acid biosynthesis genes in a wide diversity of archaeal genomes and the possibility that some of these genes were present in the last common archaeal ancestor imply that ancient assumptions of the lack of fatty acids in archaea must be revisited. Indeed, these arguments may imply that fatty acid synthesis was inherited in archaeal organisms from their last common ancestor, and because of the similarity between the bacterial and the archaeal counterparts, it would seem reasonable to propose that this machinery was present and able to produce hydrocarbon chains in the cenancestor.

Ether bonds could have existed in the cenancestor

Once the glycerol phosphate backbones and the hydrophobic tails are synthesized, these elements are specifically linked together, typically through ether links in archaea and ester links in bacteria. As for the specific utilization of G1P and G3P discussed above, this difference has also been considered as an important distinctive feature and as argument to postulate the absence of lipid membranes in the cenancestor. However, as previously reported, many exceptions are known in a variety of modern bacteria and eukaryotes (Damste et al. 2007; Huber et al. 1992; Jung et al. 1994; Langworthy et al. 1983; Mangold and Paltauf 1983; Rutters et al. 2001; Sinninghe Damste et al. 2002; Weijers et al. 2006). From an enzymatic point of view, (*S*)-3-*O*-geranylgeranylglyceryl phosphate synthase (GGGPS), the enzyme that transfers the first isoprenoid chain to G1P in archaea (Fig. 3), has been argued to be restricted to the archaeal domain, despite the existence of some bacterial homologues interpreted as HGT acquisitions (Boucher et al. 2004). The enzyme responsible of the second ether-related isoprenoid transfer to the G1P backbone, called (*S*)-2,3-Di-*O*-geranylgeranylglyceryl phosphate synthase (DGGGPS), has also been thought to be limited to archaea (Hemmi et al. 2004). Since these enzymes catalyze the crossroad steps at which the three major characteristics of archaeal polar lipids are assembled in one molecule (G1P and isoprenoid chains bound by ether links), they have independently been proposed to have triggered the emergence of archaea (Hemmi et al. 2004; Payandeh et al. 2006). Nevertheless, both GGGPS

and DGGGPS belong to respective large families of prenyltransferases widespread in the three domains of life: GGGPS is part of a group of enzymes known to transfer isoprenoid groups to a wide diversity of non-isoprenoid acceptors (Soderberg et al. 2001), whereas DGGGPS is related to the UbiA prenyltransferase family involved in biosynthesis of diverse molecules like respiratory quinones, hemes and pigments in the three domains of life (Hemmi et al. 2004).

Although accurate phylogenies of these enzyme families would provide stronger arguments to confirm or deny the utilization of ether bonds in the cenancestor, (i) the wide dispersal of ether-linked phospholipids in organisms from the three domains of life; (ii) the apparent ubiquity of phospholipid ether-forming archaeal enzyme families; (iii) and the very large substrate range of these enzymes, provide preliminary support to the idea that ether-linked phospholipids may have a very ancient origin in the three domains of life. If information about the early evolution of ether link synthesis remains scarce, to our knowledge the analogous bacterial/eukaryotic ester-forming phosphatidic synthesis has not been studied with respect to its evolutionary origin yet.

Phospholipid head groups are conserved across the three domains of life

As we have shown, most controversies about the presence of phospholipids in the cenancestor have focused on dissimilarities between archaeal and bacterial/eukaryotic glycerol phosphate enantiomers, hydrophobic chains and bonds among them. Polar head group synthesis has been less considered because of their apparent scattered taxonomic distribution. However, first surveys suggest that at least part of polar head group biosynthesis is shared among the three domains of life and could provide interesting insights on phospholipid early evolution (Koga and Morii 2007).

Once glycerol phosphate moieties and hydrophobic chains have been linked together into archaetidic or phosphatidic acids, these intermediates are respectively activated into subsequent CDP-archaeol or CDP-diacylglycerol (Fig. 3). Analogous reactions have been described both in bacteria (Dowhan 1997) and archaea (Morii et al. 2000). The primary sequence of the archaeal enzyme has not been identified so far, but the inability to detect a homologue of the bacterial one in the genome of the archaeon *Methanothermobacter thermoautotrophicus* (Morii et al. 2000) could be interpreted as evidence of the

non-homology of the respective enzymatic mechanisms. Nonetheless, information about this step is yet too scarce to confidently infer its presence or absence in the cenancestor.

In contrast, some promising work has been carried out on the replacement of CDP by the polar head residues. Although polar groups may be very diverse in the three domains of life (Koga and Morii 2005; Morii et al. 2010), L-serine, *myo*-inositol, glycerol and ethanolamine have been demonstrated to occur in bacteria, eukaryotes and archaea (Koga and Morii 2007). The CDP-alcohol phosphatidyltransferase family is a group of homologous enzymes responsible of the polar head group link during phospholipid synthesis (Fig. 4). Enzymes from this family are well characterized in bacteria (Cronan 2003), and two archaeal representatives (archaeatidylserine synthase and archaetidyl-*myo*-inositol synthase) have already been isolated and sequenced, showing homology between the archaeal and the bacterial counterparts (Morii et al. 2009; Morii and Koga 2003). Furthermore, similarity searches carried out on archaeal genomes allowed the detection of hypothetical proteins similar to the bacterial CDP-alcohol phosphatidyltransferase family. These homologues were widespread in archaea and they could be classified into two groups related to their bacterial homologues (Daiyasu et al. 2005). Phylogenetic reconstructions of the CDP-alcohol phosphatidyltransferase family supported their ancestral presence in both bacteria and archaea, which favors the hypothesis of vertical inheritance of the polar lipid biosynthesis machinery from the cenancestor (Daiyasu et al. 2005; Koga and Morii 2007).

Beyond phospholipids: ancestral protein membrane components

The uncertainty of the presence of a lipid membrane in the cenancestor transcends the simple question of the existence of a boundary in this organism. Indeed, as contemporary membranes are major sites of cellular and metabolic essential processes, this issue also addresses our view of other functions in the cenancestor: Was it endowed with an energy-transducing system? Did it bear a metabolite transport complex able to provide necessary molecules and release waste products? Was it equipped with a protein membrane-targeting mechanism in charge of protein insertion within the phospholipid bilayer and peptide secretion?

The first membrane protein complexes that were studied with regard to their presence in the cenancestor were ATPases. Representatives from this family can either use a proton or a sodium gradient to synthesize ATP, or hydrolyze ATP to carry out solute transport

(Nakanishi-Matsui and Futai 2006). They are ubiquitous complex machineries made up, among others, of two subunit types that are mutual paralogues. As these paralogues were universally distributed, they were used to reconstruct a rooted tree of life (Gogarten et al. 1989). Consequently, these complexes are among the minimal components that are thought to have been present in the cenancestor. Now, these enzymes need hydrophobic bilayers to function and they are always related to ion gradients, so their presence in the cenancestor suggests that this organism had a lipid bilayer more or less impermeable to ions, although the nature of the ions (Na^+ or H^+) has been recently called into question (Mulkidjanian et al. 2009; Mulkidjanian et al. 2008).

Other noteworthy membrane systems related to energy metabolism are the respiratory chains. In modern cells, respiratory chains transduce energy by the transfer of electrons from a donor to a final acceptor through membrane-associated redox chains, and couple it to proton translocation from one side of the membrane to the other. Many different respiratory chains are currently known to take place in very diverse organisms. Four of them have been analyzed and proposed to have been present in the cenancestor based on their widespread distribution in archaea and bacteria (Castresana and Moreira 1999). Aerobic respiration has been the most debated one, since the presence of an O_2-consuming pathway in the cenancestor seemed contradictory with the assumption that O_2 was absent in the early atmosphere and became highly concentrated much later thanks to cyanobacterial oxygenic photosynthesis (Klein et al. 1992). Nevertheless, localized traces of oxygen may have existed in the early atmosphere due to water photolysis (Kasting 1993) and recent detailed phylogenetic analyses support that one of the known dioxygen reductases (A- O_2Red) predated the divergence of the three domains of life (Brochier-Armanet et al. 2009). In the same way, nitrate, sulfate and sulfur respiration were also proposed to have been present in the cenancestor based on the occurrence of genes coding the proteins involved in electron transfer to inorganic compounds (the terminal acceptors) in a wide bacterial and archaeal diversity (Castresana and Moreira 1999), although in some cases controversies have raised about the possibility of frequent HGTs instead of the inheritance from the cenancestor (e.g. see the debate about sulfate respiration in Fukuba et al. 2003; Klein et al. 2001; Larsen et al. 1999; Wagner et al. 1998). Apart from these controversies, it is noteworthy that the intermediate ubiquitous electron carriers shared by respiratory chains (as for example cytochrome b, the Rieske protein, the blue copper protein, 2F-2S ferredoxin, 4Fe-4S ferredoxin, rubredoxin and flavodoxin), have also been proposed to be ancestral to all living organisms based on the detection of their homologues in a wide

diversity of genomes (Castresana and Moreira 1999), and supplementary data and phylogenies have provided additional support to several of these assumptions (Baymann et al. 2003; Lebrun et al. 2006; Vignais et al. 2001). Therefore, according with the accumulation of new data, the proteins belonging to the respiratory chains may have been present in the cenancestor. Since all known respiratory chains are embedded within lipid membranes, their presence in the cenancestor also supports the existence of a lipid boundary in this organism.

Besides energy metabolism, the protein targeting machinery and the protein membrane translocation systems have also attracted much interest because of their widespread distribution in modern cells and the importance of their functions. The signal recognition particle (SRP) and its SRP receptor (SR) are respectively involved in the recognition of newly synthesized peptides under elongation and the binding to their elongating ribosomes. Thus, this mechanism participates in both unfolded protein insertion into membranes and protein secretion to the outside of the cell. The major components of this system, SRP and SR, are mutual paralogues which are among the few known genes to be shared by all modern cells. As a result, they have been used, similarly to the ATPases described above, to reconstruct universal phylogenies in an attempt to root the tree of life. Their phylogenies confirmed their vertical inheritance from a common ancestor to all domains of life, what definitely supports their presence in the cenancestor (Gribaldo and Cammarano 1998).

In addition to the SRP/SR system, three protein translocation mechanisms can be predicted to have existed in the cenancestor: (i) the Sec pathway is the main machinery of protein export and membrane insertion in many bacteria, and although the archaeal counterparts share little similarity, some of them can complement their bacterial homologues (Auer et al. 1991). Moreover, phylogenies and functional comparisons support the ancestral presence of several Sec components in the cenancestor (Cao and Saier 2003); (ii) the YidC pathway is principally related to protein insertion in bacterial membranes, but it has also been detected in a wide diversity of euryarchaeotal genomes. Although functional studies on archaeal genes have not been reported yet, the phylogeny of this family of translocases was congruent with their presence in the cenancestor (Yen et al. 2001); (iii) the Tat pathway is hitherto the only translocation system known to be able to transport folded proteins across the membranes. The complex is made up of at least two subunits, the pore-forming unit TatA and the peptide-signal recognition TatC, which are present in bacteria, chloroplasts and archaea (Yuan et al. 2010). Although archaeal components of this system have only been described in some halophilic archaea (Rose et al. 2002), genomic surveys show that these elements are

present in a wide diversity of bacteria and archaea and phylogenies support an ancient origin in both lineages, what suggests that the cenancestor likely bore the components of this translocating system (Yen et al. 2002). In line with these observations, two families of signal peptidases responsible for the removal of signal sequences from the precursor proteins and likely also signal peptide peptidases that cleave signal peptides after their partition from the pre-proteins have been observed to be shared by organisms of the three domains of life (Eichler 2002; Ng et al. 2007). Thus, despite the lack of phylogenies for these enzymes, it is tempting to postulate that the structures responsible for the last steps of protein translocation were also vertically inherited in the three domains of life. Together, these results strongly support the presence of several protein membrane-targeting and translocation systems in the cenancestor, implying the existence of a membrane in this organism.

Some other protein components related to membranes have been proposed to be present in the cenancestor, as the ABC transporter family and the ion-coupled permeases (Delaye et al. 2005). However, these propositions are based on genome comparisons of only one organism from each domain of life, and consequently, further phylogenomic analyses are essential to test them. Meanwhile, a conservative proposition of the membrane proteins present in the cenancestor would include ATPases, respiratory chains and several protein membrane targeting and translocation mechanisms.

Conclusion

We have reported here a historical analysis of the evolution of the ideas concerning the presence of lipid membranes (or their absence) in the cenancestor, as well as depicted the *état de l'art* with respect to modern phylogenomic knowledge on this subject. In spite of the major efforts that have been done to answer to this capital question about the cenancestor, we have seen that many uncertainties remain.

On the one hand, the main objection to the arguments suggesting the presence of a lipid membrane in the cenancestor so far is the fact that some of them are indicative instead of demonstrative: (i) the cenancestor bore genes that may have catalyzed the synthesis of glycerol phosphate enantiomers and ether links, but this does not exclude the possibility that they carried out other functions in early evolutionary times; (ii) similarly, it is not possible to exclude that fatty acids where present in the cenancestor but current evidence does not totally

prove that they were there; (iii) some metabolite transport system could have existed in the cenancestor (ABC transporters and permeases), as well as several respiratory chains, but further phylogenomic analyses must be carried out in order to ensure this possibility. On the other hand, some indirect elements look very likely: (i) ATPases, membrane-targeting and membrane translocation mechanisms existed in the cenancestor, as well as some membrane proteins linked to respiratory chains; (ii) the isoprenoid precursors could enzymatically be produced in this organism; (iii) at least some polar head groups binding to hypothetical phospholipids can be strongly assumed to have been present at that time.

In this context, it is difficult to definitely assure the exact type of lipid membrane that could have existed in the cenancestor, what explains why this question remains a hot topic in the debates about early evolution and the nature of the cenancestor. Probably, many insights could be provided through new approaches, as for example the so-called 'enzyme resurrection', which consists in the bioinformatic inference of ancestral enzyme sequences in view of their synthesis to allow biochemical characterization (Gaucher et al. 2008; Gaucher et al. 2003; Perez-Jimenez et al. 2011).

Anyway, pending new evidence about this subject, we have shown here that scenarios invoking an acellular ancestor or inorganic boundaries in the cenancestor fail to provide a reliable explanation for the whole set of characters inferred to have existed in this organism. Lipid-free hypotheses were proposed because modern membrane components were thought to be absent in the cenancestor, but as long as more recent results cannot discard their occurrence in it, the most parsimonious hypothesis remains that the last common ancestor of all living cells was already a cell, endowed with some kind of lipid boundary, and bearing some energy-producing and translocation functions. We cannot yet provide a detailed representation of the precise chemical nature of this membrane, but the likely presence of a membrane supports that the cenancestor was already a complex organism very distant from the early steps of the origin of life. It probably had an energy transduction system, some essential metabolic pathways, the machinery for the syntheses of proteins, lipids and nucleic acids and, thus, very likely a genome of considerable size. Nevertheless, these metabolic abilities were probably less specific in the cenancestor than they are now in contemporary cells, so the noteworthy divergences among modern lineages may have been the result of optimization through time from a quite large common unspecific background, as evolution has been doing since that time.

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Fig. 1. Two types of phospholipids in the three domains of life.

Fig. 2. The boundary status in the cenancestor.

Fig. 3. Some representative steps of the phospholipid biosynthetic pathways in bacteria/eukaryotes and archaea. In bacteria and eukaryotes: (1) G-3-P dehydrogenase; (2) G3P acyltransferase; (3) 1-acylglycero-3-phosphate acyltransferase; (4) CDP-diacylglycerol synthase; (5) CDP alcohol phosphatidyltransferase. In archaea: (1) G1P dehydrogenase; (2) (S)-3-O-geranylgeranylglyceryl phosphate synthase; (3) (S)-2,3-Di-O-geranylgeranylglyceryl phosphate synthase; (4) CDP-archaeol synthase; (5) archeatidylserine synthase (and other homologues with related functions).