Journal of Membrane Biology 249 (2016) 251-259

Stimulation of Na^+,K^+-ATPase activity as a possible driving force in cholesterol evolution

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Abstract

Cholesterol is exclusively produced by animals and is present in the plasma membrane of all animal cells. In contrast, the membranes of fungi and plants contain other sterols. To explain the exclusive preference of animal cells for cholesterol we propose that cholesterol may have evolved to optimise the activity of a crucial protein found in the plasma membrane of all multicellular animals, namely the Na^+,K^+-ATPase. To test this hypothesis mirror tree and phylogenetic distribution analyses have been conducted of the Na^+,K^+-ATPase and 3β-hydroxysterol Δ^{24}-reductase (DHCR24), the last enzyme in the Bloch cholesterol biosynthetic pathway. The results obtained support the hypothesis of a co-evolution of the Na^+,K^+-ATPase and DHCR24. The evolutionary correlation between DHCR24 and the Na^+,K^+-ATPase was found to be stronger than between DHCR24 and any other membrane protein investigated. The results obtained, thus, also support the hypothesis that cholesterol evolved together with the Na^+,K^+-ATPase in multicellular animals to support Na^+,K^+-ATPase activity.

Keywords: sodium pump; plasma membrane; eukaryote; 3β-hydroxysterol Δ^{24}-reductase; squalene monooxygenase; mirror tree analysis

Introduction

The molecule cholesterol has a very bad reputation. Even amongst the general public it is now common knowledge that high blood cholesterol levels are an important risk factor for the development of cardiovascular disease. Indeed, cholesterol has such a bad name that food companies even use the label “no cholesterol” as a marketing ploy on olive oil, sugar and many other products which have no reason to contain cholesterol in the first place. However, cholesterol is present in animal plasma membranes to a level of approximately 30 – 50 mol% (van Meer et al. 2008; Cornelius et al. 2015), and animals in fact synthesize cholesterol via multi-step enzymatic pathways. Therefore, animals must produce cholesterol for some good reason. In many animals, cholesterol is an intermediate in the production of steroid hormones and bile salts. However, this cannot explain the purpose of the high level of cholesterol in their plasma membranes. This question has puzzled physiologists, biochemists and biophysicists for decades.

In an interesting hypothesis, Bloom and Mouritsen (1988) proposed that the incorporation of sterols in the plasma membrane removed a bottleneck in eukaryotic evolution. The synthesis of sterols requires the presence of oxygen, which didn’t occur in large amounts in the Earth’s atmosphere until more than 2.4 billion years ago (Mouritsen and Zuckermann 2004; Galea and Brown 2009), when the proliferation of photosynthetic cyanobacteria massively increased the O_2 level. Based on physical measurements, Bloom and Mouritsen (1988) suggested that the presence of sterols in the membrane enables it to vary its hydrophobic thickness so as to match that of integral membrane proteins as well as to maintain its fluidity and integrity over a wide temperature range. They also supported an earlier hypothesis of Cavalier-Smith (1975), who proposed that a flexible plasma membrane was crucial to the development of the processes of endo- and exocytosis, which enable eukaryotic cells to import or export material, or even engulf other entire cells, e.g.

prokaryotes, as envisaged by the endosymbiotic hypothesis of eukaryotic development (Sagan 1967). It seems entirely reasonable that the development of cytotic processes would significantly increase the rate of evolution of new species. Although these hypotheses are very attractive, the question remains as to why animal plasma membranes contain cholesterol and not some other sterol. Cholesterol is not a ubiquitous eukaryotic molecule; it is a specific animal molecule (Nes 1974). In the plasma membranes of other eukaryotes, such as fungi and plants, ergosterol and β-sitosterol are the major sterols found (Mouritsen and Zuckermann 2004; Galea and Brown 2009).

In another interesting hypothesis, Galea and Brown (2009) suggested that sterols may have evolved partially as an adaptation to the rise of oxygen in the atmosphere, not just because oxygen is required for their synthesis. O_2 is a very reactive molecule which can cause a vast range of oxidative damage to living organisms. Therefore, numerous protective measures are required. Galea and Brown (2009) proposed that sterols could protect against oxidative damage by reducing the membrane permeability towards O_2 and/or reacting themselves with O_2 to form oxysterols. Their hypothesis was based in part on the experimental observation that animals reduce their biosynthesis of cholesterol when placed on a cholesterol-rich diet (Schoenheimer and Breusch 1933). Thus, a negative feedback mechanism must exist in cholesterol biosynthesis. Galea and Brown (2009) proposed that negative feedback was due to a cholesterol-induced reduction in the entry of O_2 into the cell and hence a reduction in the rate of cholesterol synthesis. This also seems like an attractive and entirely plausible hypothesis. However, as in the case of the hypothesis of Bloom and Mouritsen (1988), it doesn't explain the specific requirement of animal cell membranes for cholesterol and not some other sterol.

Considering now the physical effects of cholesterol on a membrane, the origin of most effects would seem to be due to a preferential intermolecular interaction between cholesterol

and the hydrocarbon chains of saturated phospholipids. This leads to an increase in the order of the chains and consequent increases in the membrane thickness and the membrane dipole potential (Szabo 1974; Yeagle 1985; Starke-Peterkovic et al. 2006). These cholesterol-induced changes would be expected to cause a general decrease in permeability of the membrane towards small molecules, but particularly towards small hydrophilic cations. However, these effects are observed to occur to a greater or lesser extent in the presence of a variety of sterols, not purely cholesterol (Henriksen et al. 2006; Cournia et al. 2007). Therefore, the peculiar preference of animal plasma membranes for cholesterol remains obscure.

In the light of this, a possibility which has occurred to us is that the origin of the exclusive presence of cholesterol in animal plasma membranes may lie in an interaction with a specific protein which occurs predominantly in animal membranes and that the structure of cholesterol has been optimized in the course of evolution to interact with this protein. A prime candidate would appear to be the Na^+,K^+-ATPase, which, like cholesterol, is present in all animal plasma membranes (Haines 2001). In fact, the possession of a Na^+,K^+-ATPase could even be used as a criterion for the classification of an organism as an animal. Haines (2001) has suggested that the preference of animal plasma membranes for cholesterol could be due to its ability to reduce the membrane permeability towards Na^+, which would reduce the ATP expenditure by the Na^+,K^+-ATPase, necessary to maintain the Na^+ electrochemical potential gradient across the membrane, and hence make the cell more energy-efficient. This would be an indirect coupling between Na^+,K^+-ATPase activity and cholesterol via ATP and the Na^+ gradient. However, other results indicate a much more direct interaction. Measurements of Na^+,K^+-ATPase activity in both natural membrane preparations (Yeagle et al. 1988) and in reconstituted vesicles (Cornelius 2001) indicate that at physiological levels (around 40 mol % of the total lipid) cholesterol produces a massive activation of the protein's

activity, suggesting that cholesterol is essential for proper physiological function of the Na^+,K^+-ATPase. Furthermore, recent x-ray crystal structures show that cholesterol co-crystallises with the protein (Shinoda et al. 2009; Laursen et al. 2013; Kanai et al. 2013). Whether it is the specifically bound cholesterol molecules or ones in the neighbouring membrane which are responsible for the activation of the Na^+,K^+-ATPase is still unclear. Nevertheless, the proposition that cholesterol may have evolved in animal membranes because of its interaction with the Na^+,K^+-ATPase seems to be a plausible hypothesis which deserves further investigation.

Briefly, it is interesting to consider why all animal cell membranes contain the Na^+,K^+-ATPase. It is generally accepted that the first animals evolved in the oceans where there is a high concentration of Na^+. Thus, unicellular animal precursors would have naturally had a high concentration of extracellular Na^+. For multicellular animal organisms to maintain the same "seawater"-like extracellular milieu when direct contact to the sea was no longer possible because of multicellular organisation, a sodium pump was required (Rossier et al. 2015). The Na^+,K^+-ATPase probably evolved from earlier H^+-pumping P-type ATPases of unicellular organisms, but it is most likely that the ancestral Na^+,K^+-ATPase evolved before the split between the eukaryotic kingdoms of fungi and animals, because there are many fungi which also possess Na^+,K^+-ATPases (Sáez et al. 2009). Na^+,K^+-ATPases are also found in some protozoans, i.e., unicellular eukaryotic organisms with some animal-like characteristics (e.g. motility) and which in the past were classified as part of the animal kingdom. However, the majority of protozoans don't possess a Na^+,K^+-ATPase, whereas all metazoans (true multicellular animals) do. Thus, it seems clear that the requirement of animals for a Na^+,K^+-ATPase is connected to multicellularity.

In this paper, our aim is to investigate whether the evolution of cholesterol may have been driven by the evolution of the Na^+,K^+-ATPase and the requirement of this enzyme of a

molecule such as cholesterol to optimise its activity. Because cholesterol is a small molecule, not a protein, this question can't be tackled directly using available protein sequence data. However, cholesterol is produced by two parallel biosynthetic routes, termed the Bloch and Kandutsch-Russell pathways, both beginning with the cholesterol precursor lanosterol. The terminal enzyme in the Bloch pathway is 3β-hydroxysterol Δ^{24}-reductase (DHCR24) (Zerenturk et al. 2013). In the Kandutsch-Russell pathway the same enzyme is present, but there it is considered to catalyse the first rather than the last reaction step of the pathway. Recent results, however, indicate that in many tissues a hybrid between the Bloch and Kandutsch-Russell pathways is followed. However, regardless of the precise pathway, DHCR24 always catalyses one of the reaction steps, with its position in the sequence of reactions merely varying (Mitsche et al. 2015). Therefore, an analysis of the co-evolution of DHCR24 and the Na^+,K^+-ATPase could reveal whether or not cholesterol evolved in parallel with the Na^+,K^+-ATPase. An advantage of this approach is that it explicitly excludes the consideration of organisms which either haven't evolved or have lost the capacity to synthesize cholesterol, but still have it in their plasma membranes through their dietary intake.

Methods

Mirror-tree analysis

To analyse the degree of co-evolution of different proteins we have used the Mirrortree web server developed by Ochoa and Pazos (2010). Details of the server's workflow are described elsewhere (Ochoa and Pazos 2010). Amino acid sequences of pairs of proteins of interest (e.g. the Na^+,K^+-ATPase and DHCR24) were obtained either from the PDB or Uniprot databases and input to the server, which estimates a phylogenetic tree for each family of proteins. The trees are inferred using all available homologous sequences across the tree of

life. Comparisons between the trees of each protein can be carried out on the complete trees or subsets thereof, for example, in our case, all eukaryotes (Eukaryota), just animals, fungi and choanoflagellates (Opisthokonta), or just true multicellular animals (Metazoa). The server quantifies the degree of co-evolution of the two proteins by the degree of similarity of the trees, calculated by comparing the evolutionary distances between pairs of species which both express each protein (Pazos and Valencia 2001). The evolutionary distance is defined as the sum of the lengths of each branch of the tree separating the two species. A correlation coefficient, r_{AB}, between the evolutionary distances of each tree is calculated via the method of Pearson, as described by the following equation:

$$r_{AB} = \frac{\sum_{i=1}^{n-1}\sum_{j=i+1}^{n}\left(dA_{ij} - \overline{dA_{ij}}\right)\left(dB_{ij} - \overline{dB_{ij}}\right)}{\sqrt{\sum_{i=1}^{n-1}\sum_{j=i+1}^{n}\left(dA_{ij} - \overline{dA_{ij}}\right)^2}\sqrt{\sum_{i=1}^{n-1}\sum_{j=i+1}^{n}\left(dB_{ij} - \overline{dB_{ij}}\right)^2}} \qquad (1)$$

where dA_{ij} is the distance between species i and j on the tree of protein A. dB_{ij} is the corresponding distance on the tree of protein B. The terms with bars represent the average values of dA_{ij} and dB_{ij} across all pairs of species common to both trees. n is the number of pairings of the common organisms (or the number of elements in a matrix of the common organisms). n is related to the number of common organisms, N, by $n = (N^2 - N)/2$. The closer the value of r_{AB} is to 1, the higher the likelihood that the two proteins co-evolved. More precisely, if there is a high correlation, it means that there is a tight relationship between changes in evolutionary rates for the two families of proteins along different branches of the tree of life. For example, if one protein experiences a rate increase along one branch, then the other protein also experiences a rate increase along the same branch. Shared patterns of rate variation suggest that the proteins have evolved in a correlated manner, perhaps due to similar adaptive pressures.

To compare correlation coefficients obtained for different pairs of proteins we first converted the r_{AB} values using Fisher's *r*-to-*z* transformation to obtain a normal distribution. The Fisher *z* coefficient is related to r_{AB} by:

$$z = \frac{1}{2}\ln\left(\frac{1+r_{AB}}{1-r_{AB}}\right) \qquad (2)$$

The probability of the null hypothesis, *P*, that two correlation coefficients are not significantly different was then calculated from the corresponding *z* values and the associated number of pairs of organisms, *N*, using the on-line Vassarstats Website for Statistical Computation (http://vassarstats.net/). Analysis of individual correlation coefficients were also carried out using the Vassarstats Website. In this case *P* represents the probability of the null hypothesis that there is no significant correlation between the evolutionary distances of the two proteins being compared.

Phylogenetic distribution analysis

A further indication of co-evolution of two proteins is their co-occurrence across a large number of organisms (de Juan et al. 2013). Therefore, to further analyse whether correlations in evolutionary distances between two protein families identified via the mirror tree analysis could be attributed to a functional co-evolution we have carried out an analysis of the phylogenetic distributions of each protein family. Phylogenetic distribution can simply be expressed as the percentage occurrence of a protein across different phylogenetic classes of organisms. The phylogenetic distributions were calculated based on the phylogenetic trees constructed by using the Mirrortree server. In the case of the Na^+,K^+-ATPase, only organisms which could be clearly identified as expressing the catalytic α-subunit of the enzyme were used in deriving the phylogenetic distribution. All calculated distributions must be considered to be apparent ones, because they are limited by the available protein sequence data.

Results

Correlation of the Na^+,K^+-ATPase with squalene monooxygenase

Squalene monooxygenase is the enzyme which introduces the first oxygen atom in the sterol biosynthetic pathway (Galea and Brown 2009). Thus, it is necessary for the synthesis, not only of cholesterol, but also for ergosterol (in fungi), phytosterols (in plants) and lanosterol (produced at an early stage of sterol biosynthesis and found in some nematodes). Because squalene monooxygenase is so widely distributed, one would not expect it's evolution to be linked to that of the Na^+,K^+-ATPase, which is predominantly an animal enzyme. To test this the amino acid sequence of human squalene monooxygenase (Uniprot Q14534) was submitted to the Mirrortree server together with sequences of the Na^+,K^+-ATPase.

Within the group Opisthokonta, i.e. animals and fungi, the correlation coefficients found were as follows: 0.581 (N = 61, PDB 2ZXE), 0.665 (N= 60, PDB 4HQJ), 0.562 (N = 61, PDB 3B8E), and 0.596 (N = 62, PDB 3WGU). Within the animal kingdom alone (Metazoa) a reliable analysis wasn't possible because there is an insufficient number of common organisms (N = 6). Most of the organisms possessing both squalene monooxygenase and a Na^+,K^+-ATPase were in fact fungi. Actually there are likely to be many more common organisms, but the analysis is restricted to organisms for which the amino acid sequence of both squalene monooxygenase and the Na^+,K^+-ATPase are available. The r_{AB} values found for the two proteins within Opisthokonta indicate a relatively poor correlation.

Correlation of the Na^+,K^+-ATPase with DHCR24

The correlation coefficients for co-evolution of the catalytic α-subunit of the Na^+,K^+-ATPase and DHCR24 (Uniprot Q15392) for different taxonomic groupings are given in Tables 1 and 2. The calculations were carried out using either the amino acid sequence of shark Na^+,K^+-

ATPase, PDB 2ZXE (Shinoda et al. 2009) (Table 1) or pig Na^+,K^+-ATPase, PDB 3WGV (Kanai et al. 2013) (Table 2). The pig Na^+,K^+-ATPase sequence was supplemented by the propeptide sequence MGKGV from the PDB entry 4HQJ (Nyblom et al. 2013). Calculations with the sequence from PDB 4HQJ were also carried out, and these gave almost identical results to those shown in Table 2. However, because a small number of errors in the sequence used in PDB 4HQJ have been identified (Henriksen et al. 2013), in the case of pig Na^+,K^+-ATPase we only present the results obtained using PDB 3WGV. The values of the correlation coefficients were calculated according to eq. 1 (see Methods).

Taxonomic Group	r_{AB}	N
Complete trees	0.773	18
Eukaryota	0.791	17
Opisthokonta	0.811	16
Metazoa	0.948	11

Table 1: Pearson correlation coefficients, r_{AB}, for co-evolution of the Na^+,K^+-ATPase and DHCR24. The correlations were calculated starting with the amino acid sequence of shark Na^+,K^+-ATPase (PDB 2ZXE) (Shinoda et al. 2009). In every case the probability, *P*, of the null hypothesis that there is no significant correlation was <0.0001. The taxonomic groupings are: Eukaryota (all eukaryotes), Opisthokonta (fungal and animal eukaryotes), and Metazoa (all animals).

Taxonomic Group	r_{AB}	N
Complete trees	0.777	17
Eukaryota	0.833	15
Opisthokonta	0.852	14
Metazoa	0.941	10

Table 2: Pearson correlation coefficients, r_{AB}, for co-evolution of the Na^+,K^+-ATPase and DHCR24. The correlations were calculated starting with the amino acid sequence of pig Na^+,K^+-ATPase (PDB 3WGV) (Kanai et al. 2013). In every case the probability, *P*, of the null hypothesis that there is no significant correlation was <0.0001. The taxonomic groupings are the same as listed in Table 1.

The data clearly show a continuous gradual increase in the correlation as one moves from the complete trees, through eukaryotes in general, multicellular animals and fungi, and finally to animals alone. The correlations for Metazoa are shown graphically in Fig. 1.

Within the Metazoa, the cholesterol molecule itself has already evolved and its structure is static. Therefore, the observed correlation in co-evolution between the Na^+,K^+-ATPase and DHCR24 cannot be due to an evolution of cholesterol synthesis. The correlation could, however, be attributed to a continuing evolution in the kinetics of cholesterol synthesis via DHCR24 or to mutations of the DHCR24 amino acid sequence affecting the regulation of cholesterol biosynthesis.

The common animal species giving the points shown in Fig. 1 are mouse, rat, human, cow, chicken, zebrafish (*Danio rerio*), green spotted pufferfish (*Tetraodon nigroviridis*), yellow fever mosquito (*Aedes aegypti*), two species of nematode (*Caenorhabiditis elegans*

and *Caenorhabiditis biggsae*), and the starlet sea anemone (*Nematostella vectensis*). It is clear that there would be many more animal species than this which express both proteins. However, the analysis is limited by the number of species in which both proteins have been sequenced. Nevertheless, even with the limited number of available species, there appears to be a clear correlation between the evolution of both proteins.

One needs to be aware, however, that the statistical calculation of correlation coefficients assumes at least the possibility of an underlying independence of data points. In fact, for species comparisons, as carried out here, this is not always the case, because species share parts of their evolutionary history. This is termed phylogenetic non-independence (Felsenstein 1985; Lanfear et al. 2010). For this reason it is desirable to carry out the analysis on divergent species, which only share a small portion of their evolutionary history. The Mirrortree server does not carry out the calculation of any correlation coefficients unless there are at least ten common organisms between the two protein families (Ochoa et al. 2015). Nevertheless, one needs to be wary in viewing the absolute values of correlation coefficients calculated by the server. Rather than look at the value of r_{AB} in isolation, one needs to compare the r_{AB} values determined for different pairs of protein families within the same taxonomic grouping and observe whether the correlation increases or decreases in strength.

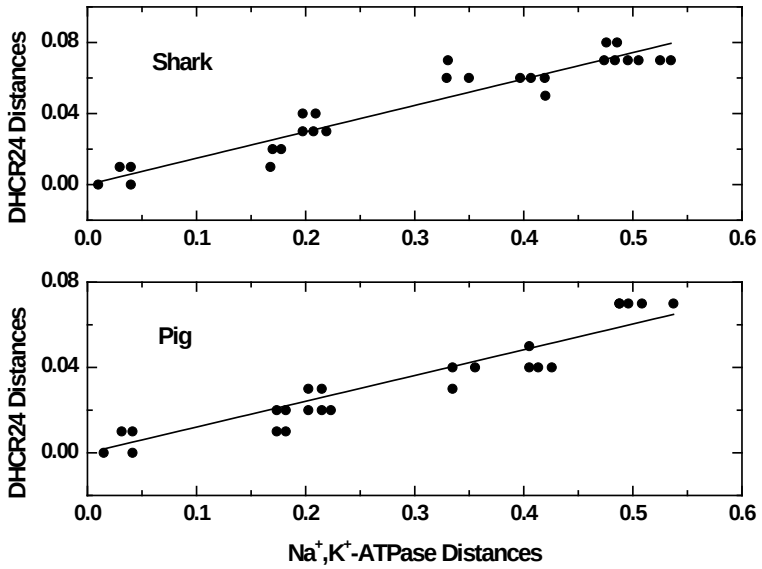

Figure 1: Correlations of the evolutionary distances between common organisms expressing DHCR24 and the Na^+,K^+-ATPase within the Metazoa, i.e., animals. The correlations were derived using the Na^+,K^+-ATPase amino acid residue sequence of shark (above) or pig (below). The solid lines represent fits of straight lines through the origin to the data. Each data point represents a combination of a pair of common organisms for each protein, as explained by eq. 1 in Methods. Hence there are more data points than common organisms.

Amongst the protein sequences showing homology with DHCR24 identified by the Mirrortree server, there was only one belonging to a prokaryote. This was a flavin adenine dinucleotide (FAD) binding protein of the bacterium *Methylcoccus capsulatus*. However, it has previously been suggested that this organism obtained another protein of the sterol biosynthetic pathway via lateral transfer of genetic material from a eukaryote (Chen et al. 2007). Therefore, it's possible that this may also be the case for the DHCR24-homologous protein. All other protein sequences in the DHCR24 phylogenetic tree were eukaryotic. Two belonged to plants, five to fungi, one to a choanoflagellate (colony-forming unicellular

eukaryotes from which animals are thought to have evolved) and eleven to animals. Therefore, as in the case of the Na^+,K^+-ATPase, DHCR24 is predominantly an animal enzyme. The function of most of the eukaryotic but non-metazoan proteins which showed homology to DHCR24 is as yet undetermined. However, many contain FAD binding domains, which would lead one to suspect that they are likely to be redox enzymes, as is DHCR24. The homologous protein of the plant *Arabidopsis thaliana* has been identified as a Δ^{24} sterol reductase, but which catalyses the formation of a plant sterol rather than cholesterol (Waterham et al. 2001).

A mirror-tree analysis on 3β-hydroxysterol Δ^7-reductase (DHCR7, Uniprot Q9UBM7), the penultimate enzyme of the Bloch pathway and the final enzyme of the Kandutsch-Russell pathway (Zerenturk et al. 2013; Mitsche et al. 2015), and the Na^+,K^+-ATPase (shark sequence PDB 2ZXE) was also carried out. In that case, however, the correlation was very poor. The results obtained were as follows: Complete trees (r_{AB} = 0.303, N = 68), Eukaryota (r_{AB} = 0.301, N = 64), Opisthokonta (r_{AB} = 0.283, N = 55) and Metazoa (r_{AB} = 0.441, N = 8). From the numbers of common organisms it is apparent that, although DHCR7 is a predominantly eukaryotic enzyme, in contrast to DHCR24 it has a wide distribution amongst non-metazoan eukaryotes, i.e., it is not predominantly an animal enzyme.

Comparisons of correlation coefficients between membrane proteins

From the analysis presented already there would appear to be a strong correlation between the evolution of DHCR24 and the evolution of the Na^+,K^+-ATPase. This would seem to support the hypothesis that cholesterol evolved to optimize the activity of the Na^+,K^+-ATPase. However, there are many other proteins in animal membranes apart from the Na^+,K^+-ATPase. Therefore, to test how unique the correlation is between DHCR24 and the Na^+,K^+-ATPase, we have also analysed the correlation between DHCR24 and other membrane-bound or -

associated proteins which are widely found in the animal kingdom as well as other kingdoms. The results, including the relevant apparent *P* values, are shown in Table 3. It is important to bear in mind that the *P* values are apparent, because phylogenetic non-independence cannot be totally excluded (see earlier). Therefore, the *P* values must be viewed only as indicative of trends. Analyses were in fact attempted using many other membrane proteins in addition to those listed in Table 3. However, in all of these cases the number of common organisms with available amino acid sequences for both the membrane protein concerned and DHCR24 was too small to allow a meaningful statistical analysis.

The correlation between the evolution of the Na^+,K^+-ATPase and DHCR24 is stronger than all of the other membrane proteins investigated. At a probability level, *P*, of 0.05 the correlation between the DHCR24 and the Na^+,K^+-ATPase appears to be significantly stronger than that between DHCR24 and cytochrome c, CoQ-cytochrome c reductase, NADH-CoQ reductase, and aquaporin. There is less confidence that the co-evolution of DHCR24 and the Na^+,K^+-ATPase is stronger than that of DHRC24 and the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase or cytochrome c oxidase. A problem here is the limited number of organisms in which both DHCR24 and the listed membrane proteins have been identified. Once more sequence data becomes available and the data set increases, more definite conclusions will become possible. Nevertheless, the data shown in Table 3 seems to indicate that there is at least a 62% probability that the evolution of DHCR24 and the Na^+,K^+-ATPase are more strongly linked than all of the other proteins investigated. Based on the shark Na^+,K^+-ATPase sequence alone, the probability increases to at least 85%.

Protein	r_{AB}	N	P_{shark}	P_{pig}
Cytochrome c	0.862	11	0.0013	0.0093
CoQ-cytochrome c reductase	0.834	11	0.0002	0.0019
SR Ca^{2+}-ATPase	0.926	11	0.1211	0.3524
NADH-CoQ reductase	0.863	11	0.0014	0.0099
Cytochrome c oxidase	0.924	9	0.1527	0.3789
Aquaporin	0.813	11	<0.0001	0.0006

Table 3: Pearson correlation coefficients, r_{AB}, for co-evolution of various membrane-bound or –associated proteins and DHCR24. N is the number of organisms. The number of points in the co-evolution plot, n, is given by $(N^2 - N)/2$, i.e., $n = 55$ for $N = 11$ and $n = 36$ for $N = 9$. All calculations were performed for the animal kingdom, i.e., Metazoa. P_{shark} and P_{pig} represent the apparent probabilities of the null hypothesis that the co-evolution correlation between DHCR24 and the listed proteins isn't significantly different from the correlation between the Na^+,K^+-ATPase of shark ($r_{AB} = 0.948$, $N = 11$, $P < 0.0001$) or pig ($r_{AB} = 0.959$, $N = 11$, $P < 0.0001$) and DHCR24.

Although the correlation observed between DHCR24 and the SR Ca^{2+}-ATPase is lower than that between DHCR24 and the Na^+,K^+-ATPase, the correlation is still relatively strong, indicating that cholesterol may also play an important role in regulating the activity of the SR Ca^{2+}-ATPase. The sarcoplasmic reticulum membrane is known to contain a lower amount of cholesterol of approximately 13 mol% (van Meer et al. 2008) relative to the plasma membrane, where the Na^+,K^+-ATPase is located, with up to 50 mol%. However, if the interaction between the SR Ca^{2+}-ATPase and cholesterol is via a non-specific membrane-

mediated mechanism, as recent molecular dynamics simulations suggest (Autzen et al. 2015), the different mole percentages of cholesterol in the plasma and sarcoplasmic reticulum membranes could be taken to indicate that cholesterol composition of each membrane has also evolved to provide a membrane with the optimum physical properties (i.e., thickness, flexibility) to suit the ion pump which it contains.

The data shown in Table 3 also show that within the Metazoa there is a relatively high correlation between DHCR24 and the cytochrome c oxidase, the final membrane protein of the electron transport chain, which is situated in the inner mitochondrial membrane. Does the observed correlation indicate that cholesterol co-evolved with the cytochrome c oxidase or that cholesterol is important for cytochrome c oxidase activity? To answer this question it is important to look at phylogenetic distributions as a further indicator of co-evolution.

Comparisons of the phylogenetic distributions of DHCR24, the Na^+,K^+-ATPase and cytochrome c oxidase

Phlylogenetic distributions of DHCR24, the Na^+,K^+-ATPase and cytochrome c oxidase, based on phylogenetic trees constructed using the Mirrortree server, are shown in Fig. 2. In comparison to the phylogenetic distribution of the cytochrome c oxidase, the distributions of DHCR24 and the Na^+,K^+-ATPase can be considered to be similar. Both are predominantly eukaryotic proteins, to 95% in the case of DHCR24 and 73% in the case of the Na^+,K^+-ATPase. Within eukaryotes, both are predominantly present amongst Fungi and Metazoa. In comparison, the cytochrome c oxidase shows the largest representation amongst bacteria. The cytochrome c oxidase is present in any organism which undergoes respiration, synthesizing ATP as the end product of the electron transport chain. Therefore, in spite of the fact that DHCR24 and cytochrome c oxidase show a correlation via the mirror tree analysis, the differences in phylogenetic distribution clearly indicate that they definitely did not evolve

at the same point in time. Nevertheless, the observed mirror tree correlation with Metazoa (see Table 3) suggests that the low level of cholesterol present within the mitochondrial membrane of animals of around 9 mol% (Van Meer et al. 2008) could be responsible for some modulation of their cytochrome c oxidase activity.

Returning to the phylogenetic distributions (see Fig. 2), DHCR24 shows a higher distribution of 55% amongst Metazoa than the Na^+,K^+-ATPase, for which the value is 31%. The Na^+,K^+-ATPase, on the other hand, shows a higher distribution amongst Bacteria and Archaea than DHCR24. This could be taken to indicate that the Na^+,K^+-ATPase evolved at an earlier stage of evolution than DHCR24 (and cholesterol), with later evolution of DHCR24 optimising the sterol-Na^+,K^+-ATPase functional relationship.

The sarcoplasmic reticulum Ca^{2+}-ATPase, which also shows a strong correlation with DHCR24 via the mirror tree analysis (see Table 3), occurs exclusively amongst eukaryotes (77.8% Metazoa and 22.2% Alveolata). This is in fact to be expected, because the sarcoplasmic reticulum Ca^{2+}-ATPase is involved in Ca^{2+} handling in muscle cells. Therefore, one would expect it to be present only in higher organisms possessing specialized tissue for motility. Its phylogenetic distribution is, thus, much more restricted than that of both DHCR24 and the Na^+,K^+-ATPase. For example, in contrast to DHCR24 and the Na^+,K^+-ATPase, it doesn't occur at all amongst plants, fungi or bacteria. Therefore, even though the mirror tree analysis yields a high correlation coefficient between the sarcoplasmic reticulum Ca^{2+}-ATPase and DHCR24, on the basis of their very different phylogenetic distributions, as in the case of the cytochrome c oxidase, it can be excluded that the two proteins evolved at the same point in time.

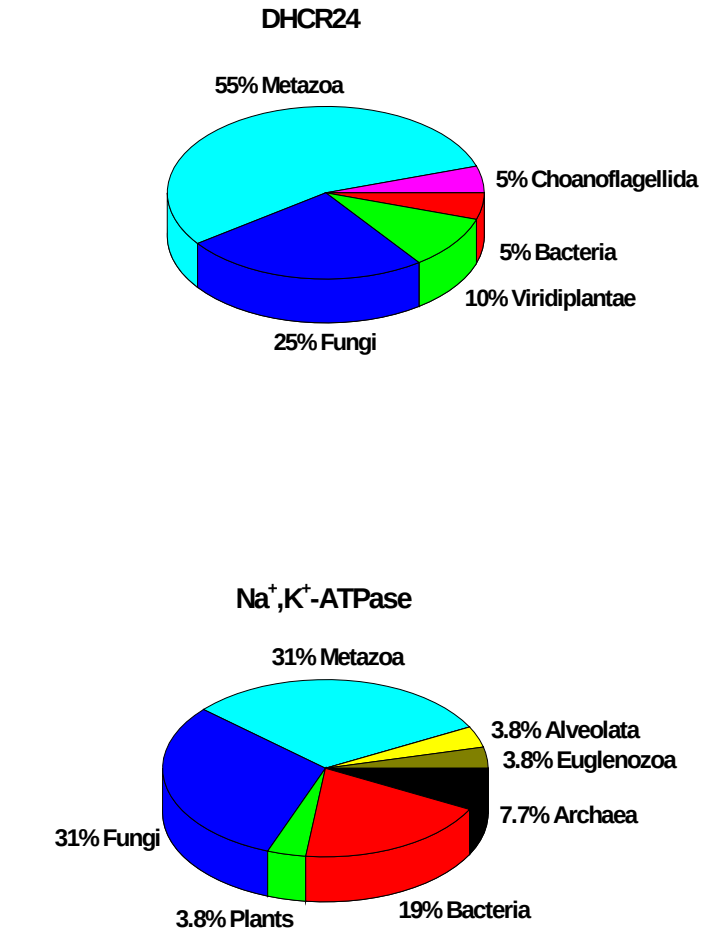

Figure 2: Phylogenetic distributions of DHCR24, the Na^+,K^+-ATPase and the cytochrome c oxidase. The distributions were calculated based on the phylogenetic trees derived from the

Mirrortree server. In the case of the Na^+,K^+-ATPase, only organisms known to express the alpha subunit of the Na^+,K^+-ATPase were included in the analysis.

Discussion

The mirror tree analysis presented here indicates that there is a relatively strong correlation between the evolution of the Na^+,K^+-ATPase and DHCR24, the terminal enzyme in the biosynthetic pathway producing cholesterol. The correlation is stronger than for any other membrane protein from the relatively limited data set investigated, which is determined by the number of organisms for which amino acid sequences have been determined for both pairs of proteins. This result is also supported by phylogenetic distribution analysis, showing a similar distribution of DHCR24 and the Na^+,K^+-ATPase. Together the results support the hypothesis that the Na^+,K^+-ATPase and cholesterol co-evolved within the animal kingdom. Furthermore, they also support the idea that a reason for the exclusive presence of cholesterol in animal cell membranes as opposed to bacterial, plant or fungal membranes is that it is required to optimise Na^+,K^+-ATPase activity. This doesn't mean that the Na^+,K^+-ATPase is the only animal membrane protein requiring cholesterol for its optimal function. There are, no doubt, many others. However, for the evolution of the cholesterol molecule the Na^+,K^+-ATPase is particularly relevant because of its requirement at the earliest stages of animal evolution, before any cell differentiation or any tissue or organ formation occurred.

A further interesting consideration is the stage of animal development at which the Na^+,K^+-ATPase and cholesterol become crucial. If they have co-evolved within animals, one would also expect their effects on an animal's viability to be linked. Because the Na^+,K^+-ATPase creates the extracellular environment required for animal multicellularity, its activity is crucial at the very earliest stages of cell division, as mutational studies have shown (Kidder and Watson 2005). No animal could survive to birth without a functional α_1 subunit of the

Na^+,K^+-ATPase. Now considering cholesterol, mutations in the gene encoding DHCR24 lead to a defective enzyme and hence the accumulation of the precursor in the Bloch cholesterol biosynthetic pathway, desmosterol (Zerenturk et al. 2013). This rare condition, known as desmosterolosis, can allow pregnancies in humans to proceed their full term, but with death sometimes occurring within hours of birth. In mice, the condition is less severe, but this has been attributed to transplacental passage of cholesterol from the mother while still in the fetus stage, a process which is not possible in humans (Wechsler et al. 2003; Brown 2004). Blockage of the Kandutsch-Russell pathway of cholesterol biosynthesis is also known to produce severe birth defects in humans. The Smith-Lemli-Opitz syndrome (SLOS) is now known to be due to a mutation is the gene which produces the enzyme 3β-hydroxysterol Δ^7-reductase (DHCR7), the terminal enzyme of the Kandutsch-Russell pathway. In this condition 7-dehydrocholesterol accumulates in the cell membrane, rather than desmosterol (Tulenko et al. 2006). Although it appears to be less lethal than desmosterolosis, with some individuals surviving into adulthood, children with SLOS still suffer from multiple birth defects. Inhibition of enzymes earlier in the Bloch biosynthetic pathway, not even allowing the synthesis of desmosterol, are even more lethal and evidence for inhibition of cell division has in fact been demonstrated (Fernandez et al. 2005). Thus, both the Na^+,K^+-ATPase and cholesterol do seem to play an important role in cell division. This provides further indirect evidence that their evolution may be linked. The fact that desmosterolosis is not immediately lethal could imply that desmosterol can still support Na^+,K^+-ATPase activity, but not at the optimal level achievable in the presence of cholesterol in the membrane. To investigate whether or not this is the case, experiments on the effect of desmosterol on the activity of purified Na^+,K^+-ATPase-containing membrane fragments in a cell-free system would be required.

In order to further substantiate the conclusions found here it is clear that further studies would be necessary. For example, as further amino acid sequences become available, the mirror tree analyses presented here could be extended, in particular to other membrane proteins known either bind cholesterol or be sensitive to its presence (Yeagle 2014). Experimental studies could also provide valuable information, e.g. by studying the activity of various membrane proteins, including the Na^+,K^+-ATPase, in the presence of various sterols along the cholesterol biosynthetic pathway. An example of this type of study is that of Yeagle et al. (1988), which indeed showed that, in comparison to ergosterol (the major fungal sterol) and lanosterol (a cholesterol precursor), cholesterol was most effective in supporting Na^+,K^+-ATPase activity.

Acknowledgements

The authors acknowledge helpful discussions with Prof. Ole Mouritsen, Assoc. Prof. Simon Ho, Assoc. Prof. Neville Firth, Prof. Andrew Brown, and Prof. Philip Kuchel. R.J.C acknowledges, with gratitude, financial support from the Australian Research Council (Discovery Grants DP-121003548 and DP-150101112).

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