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Review

sAC as a model for understanding the impact of endosymbiosis on cell signaling ☆

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ABSTRACT

As signaling pathways evolve, selection for new functions guides the co-option of existing material. Major transitions in the history of life, including the evolution of eukaryotes and multicellularity, exemplify this process. These transitions provided both strong selection and a plenitude of available material for the evolution of signaling pathways. Mechanisms that evolved to mediate conflict during the evolution of eukaryotes may subsequently have been co-opted during the many independent derivations of multicellularity. The soluble adenylyl cyclase (sAC) signaling pathway illustrates this hypothesis. Class III adenylyl cyclases, which include sAC, are found in bacteria, including the α-proteobacteria. These adenylyl cyclases are the only ones present in eukaryotes but appear to be absent in archaeans. This pattern suggests that the mitochondrial endosymbiosis brought sAC signaling to eukaryotes as part of an intact module. After transfer to the proto-nuclear genome, this module was then co-opted into numerous new functions. In the evolution of eukaryotes, sAC signaling may have mediated conflicts by maintaining metabolic homeostasis. In the evolution of multicellularity, in different lineages sAC may have been co-opted into parallel tasks originally related to conflict mediation. Elucidating the history of the sAC pathway may be relatively straightforward because it is ubiquitous and linked to near universal metabolic by-products (CO_2/HCO_3^-). Other signaling pathways (e.g., those involving STAT and VEGF) present a greater challenge but may suggest a complementary pattern. The impact of the mitochondrial endosymbiosis on cell signaling may thus have been profound. This article is part of a Special Issue entitled: The role of soluble adenylyl cyclase in health and disease.

1. Introduction

How do signaling pathways evolve? A functional view is clearly relevant. Pathways evolve because cells or organisms that possess a particular pathway are able to collect and respond to information that is ultimately environmental. The result is a fitness advantage. A historical view, however, is also relevant. Cells and organisms are not so much designed as they are cobbled together. Evolution of novel traits may often depend on what material is available to be co-opted into a new function. This may be the case with signaling pathways as much as with any other aspect of biological adaptation.

The juxtaposition of functional and historical views suggests that signaling pathways may have been particularly likely to evolve at certain times in the history of life. Periods of rapid evolution may have been characterized by both strong selection and a plenitude of available material. The major transitions in the history of life [1,2] are particularly likely to have fostered rapid evolution for these reasons. These transitions involved an expansion of levels or units of selection [3], including the transitions from prokaryotes to eukaryotes and from unicellular to multicellular organisms. These transitions may have provided novel selective scenarios as well as an abundant new material for a creative role of natural selection.

The suggestion that major signaling pathways in eukaryotes evolved during the transition from prokaryotes is hardly controversial. Similarly, novel signaling pathways in multicellular organisms likely date from early in their history. Nevertheless, evolution also implies continuous extinction, loss, and simplification. Reconstructing the history of the evolution of signaling pathways may thus be challenging [4]. In many cases, much of the history may have disappeared, like sedimentary rock that has eroded away. Some aspects may be totally obliterated, others may be partially intact, and occasionally a nearly complete record of a mechanism and its history may be found—the molecular equivalent of Lagerstätten, exceptionally rich and detailed fossil deposits.

The soluble adenylyl cyclase (sAC) pathway may be one of the latter examples. Just as rich fossil deposits can illuminate more fragmentary remains, sAC may shed light on the evolution of other signaling pathways during major transitions in the history of life. This central point is elaborated below. First, an evolutionary framework for understanding the major transitions is outlined. With this in mind, the history of the

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sAC pathway is traced in an evolutionary hypothesis that is consistent with the available data. From this example, two principal tenets emerge: (i) signaling pathways in the proto-mitochondrion were co-opted into parallel uses in the proto-eukaryote, and (ii) these signaling pathways were then repeatedly co-opted as multicellular eukaryotes emerged. Tenets (i) and (ii) are then further examined with two other examples. While the sAC pathway may be particularly widespread because it is linked to near universal metabolic by-products (CO_2/HCO_3^-), other more fragmentary examples may nevertheless provide support for the overall perspective.

2. Evolutionary context

The endosymbiont hypothesis has been widely accepted by mainstream biology [5]. Nevertheless, the implications of endosymbiosis for eukaryotes are only now being explored and appreciated. The central question is no longer whether mitochondria are products of endosymbiosis, but rather how this endosymbiosis occurred and what its effects were [6,7]. What happens when a community of bacteria takes up residence in a host cell? How can this lead to a stable association? What legacies of these early interactions remain in modern eukaryotes? What is the relationship between eukaryotic innovations and subsequent evolutionary transitions? These are some of the questions that need to be more carefully considered.

Levels-of-selection theory [3,8] provides a framework for developing answers to these questions. This theory is built on a simple premise: any biological unit that exhibits heritable variation can evolve. These evolvable units are called units of evolution or units of selection [9]. These units include some molecules (e.g., genes, prions, mobile genetic elements), some organelles (mitochondria, plastids), cells, organisms, and populations. Notions of levels of selection date back to Darwin [10]. Of course, selection at the different levels may not be of equal strength. The group selection controversies of the 1960s are now seen as a debate about the relative strength of selection at two different levels—the organism and the population.

Conceptualizing the history of life as a history of levels of selection transitions [1,2,11] recast this debate [3]. Life can be viewed as a hierarchy of units organized like Russian dolls—genes within chromosomes, chromosomes within simple cells, simple cells with complex cells, and so on. There is thus an unexpected simplicity to the history of life. Lower-level units banded together to form groups. Mechanisms that decrease variation among the lower-level units and increase variation among the groups evolved to mediate conflicts [12]. Groups that effectively deployed these mechanisms of conflict mediation successfully emerged as higher-level units. Higher-level units, being larger, had a number of ecological advantages over the original lower-level units [13]. Subsequently, these new units could band together and repeat the process.

Two of the major transitions will be the focus here—the transition from simple (prokaryotic) to complex (eukaryotic) cells and the transition from unicellular to multicellular organisms. The former is a singular event in the history of life [14], while the latter has occurred repeatedly [15,16]. Since conflict mediation is the key to these transitions [12], subsequent to the evolution of eukaryotes conflict mediation was apparently much less challenging. The later transition would seem to be facilitated by the earlier one [6]. Generally, this suggests that a greater understanding of these transitions can be obtained by focusing on the mechanisms of conflict mediation.

What are these mechanisms? Discussions of the mitochondrial endosymbiosis have more-or-less tacitly assumed that gene transfer to the nucleus mediated conflict [17]. Yet there were two large obstacles that likely prevented gene transfer from having an impact early in the endosymbiosis [6]. The first was the difficulties associated with evolving de novo the mitochondrial protein import apparatus, without which proto-mitochondria could not lose genes. The second was that selection pressure itself would prevent gene loss, if indeed retaining those genes benefited the proto-mitochondrion. The argument thus becomes uncomfortably circular: to occur, gene loss requires relaxed selection on the lower-level units, but such relaxed selection can only be achieved by gene loss.

Further, if gene loss mediated conflicts in the evolution of eukaryotes, this transition could not have facilitated the evolution of multicellularity, since gene loss is not characteristic of this later transition. Rather, conflict mediation in multicellular organisms seems to be regulated by cell signaling pathways. As pointed out by Minelli [18]: "The dynamic equilibrium (or, better, the unceasing competition) among cells in a multicellular assemblage is mediated by cell signaling." Such signaling can limit variation among lower-level units and increase variation among higher-level units [7].

Could cell signaling have mediated conflicts arising during the evolution of eukaryotes? The key to this transition may have been metabolic signaling [6]. Governed by the biophysics of the electron transport chain (Fig. 1), the characteristics of mitochondria can vary widely depending on metabolic state [19]. While these characteristics are inherent in chemiosmotic function, surprisingly complex dynamics can nonetheless result. If substrate is limiting, the trans-membrane proton gradient becomes minimal, electron carriers become oxidized, and production of reactive oxygen species (ROS) is minimal (Fig. 1a: state 2). If substrate is available, and there is metabolic demand, substrate will be oxidized at a rapid rate. Coenzymes (NAD^+ and FAD) will be reduced and re-oxidized rapidly. Electrons will rapidly proceed through the electron carriers, reducing oxygen to water, while protons are extruded. The redox state of the electron carriers and ROS levels will be moderate (Fig. 1b: state 3). If substrate is available but there is no metabolic demand, electrons back up on the electron carriers, and the trans-membrane proton gradient becomes maximal. Coenzymes remain reduced (NADH and $FADH_2$). ATP relative to ADP levels are high. The electron carriers are reduced, and ROS formation is maximal (Fig. 1c: state 4).

State 3 mitochondria have a number of features that promote homeostasis and cooperation. Energy is converted at a rapid rate with maximal amounts of ATP produced and exported. ROS form in amounts sufficient for signaling, but not at levels that cause damage. On the other hand, features of state 4 mitochondria disrupt homeostasis and lead to conflict. For instance, low levels of ATP and high levels of ROS can trigger cell death. Mechanisms that regulate metabolic state can thus mediate levels-of-selection conflict [7].

Multicellularity evolved repeatedly in diverse eukaryotic lineages [15,16]. Each time multicellularity evolved, mechanisms of conflict mediation had to evolve as well. Co-opting material from the previous evolutionary transition, involving the mitochondrial endosymbiosis, was one way to facilitate the transition to multicellularity. Metabolic signaling may again be the key. On this basis, a prediction can be made: cell signaling pathways that regulate competition among cells in a multicellular organism (sensu Minelli [18]) may have been derived from similar pathways that regulate competition among mitochondria in eukaryotic cells, and both sets of pathways may involve metabolic signaling. Nevertheless, testing this hypothesis may be challenging because of the effects of loss, reversal, and extinction on the character states of extant organisms. Analogous to the fossil record, the history of some signaling pathways may be only partially intact (or partially described). These may or may not support the interpretation offered above. Nevertheless, either way this evidence is not decisive. More critical are considerations of the molecular Lagerstätten—those pathways that are well preserved. The first example—sAC signaling—is a remarkable example of this phenomenon.

3. The sAC pathway

Cyclic AMP (cAMP) serves as a second messenger in many organisms. Adenylyl cyclases of various kinds produce cAMP from ATP, with pyrophosphate produced as a by-product. Based on the sequences of

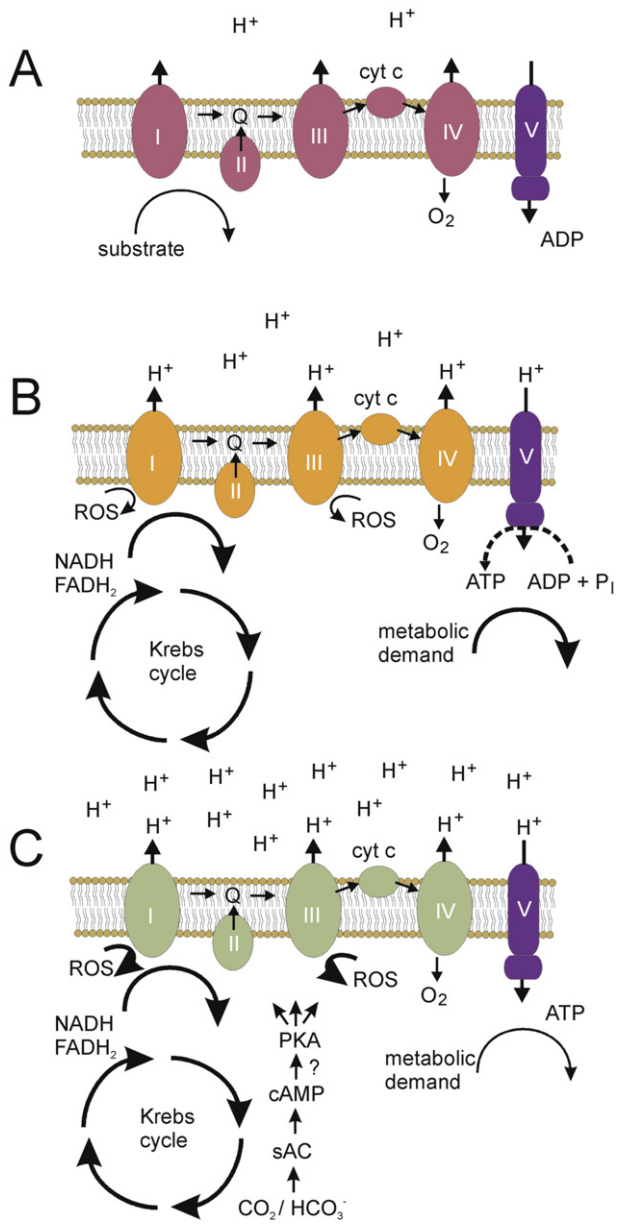

Fig. 1. Schemata of the mitochondrial electron transport chain in different redox states. Substrate is oxidized and electrons follow a path (small arrows) through the electron carriers of complexes I–IV to molecular oxygen. Protons are extruded (large arrows) and return to the mitochondrial matrix via ATP synthase (V). In state 2 (A), substrate is limiting, the redox state of the electron carriers is relatively oxidized, the trans-membrane proton gradient and reactive oxygen species (ROS) formation are both minimal, and ADP relative to ATP is maximal. In state 3 (B), substrate supply is adequate, and substrate is actively oxidized, for instance by the Krebs cycle, reducing coenzymes NAD^+ and FAD. Metabolic demand is high. The redox state of the electron carriers, the proton gradient, ROS, and ADP levels are all intermediate. Phosphorylation rates are maximal. In state 4 (C), substrate is oxidized, and coenzymes are reduced, but metabolic demand is weak. ADP relative to ATP is minimal, the proton gradient is maximal, electron carriers are highly reduced, and ROS formation is maximal. If substrate supply varies over time, a sudden increase might overwhelm the capacity of the electron transport chain to oxidize NADH and $FADH_2$, resulting in state 4 (C). Input of electrons overmatches metabolic demand, the trans-membrane proton gradient becomes maximal, the electron carriers become reduced, and reactive oxygen species (ROS) formation becomes maximal. Under these conditions, damage can occur. Signaling via CO_2/HCO_3^- (produced as a by-product of oxidation of substrate in the Krebs cycle) and sAC/cyclic AMP (cAMP)/protein kinase A (PKA) can "prime" the electron carriers for the arrival of NADH and $FADH_2$ (C). A moderate proton gradient, intermediate redox states, and low to moderate levels of ROS can then be maintained (B). Uncertainty about the mechanism by which cAMP activates respiration is indicated by the "?."

their catalytic portions, six classes of adenylyl cyclases are currently recognized [20]. Five of these classes occur only in prokaryotes. Class III adenylyl cyclases (ACs), however, are found in both prokaryotes and eukaryotes [20,21]. Indeed, these class III cyclases are widely found, including in α-proteobacteria, and both unicellular and multicellular eukaryotes. In the well-studied mammals, two types of class III ACs are found: transmembrane adenylyl cyclases and soluble adenylyl cyclases. Of the two, the latter seems most similar to those in bacteria [20]. There is some question whether bacterial forms of sAC are activated by CO_2 or HCO_3^- [22,23]; however, in most biological systems the two are inextricably linked [24]. Sporadic reports of adenylyl cyclases in archaeans have been made [25,26]. There remains some expectation that class IV ACs may yet be found in this group [27]. Nevertheless, archaeans do not seem to have the class III ACs that are so widely distributed among other organisms [27].

The roots of adenylyl cyclases may extend to the RNA world when nucleotides first became widely used by living things [28]. The adenylyl cyclase reaction could originally have run in reverse, generating ATP or GTP from cAMP or cGMP, while consuming pyrophosphate [25]. Such a reaction would convert pyrophosphate, the pre-RNA world energy currency [29], to ATP or GTP, the RNA world energy currency. Adenylyl cyclases may have had their origins as such "currency exchangers." Running in a forward direction, the initial signaling function of class III ACs may have been related to the CO_2/HCO_3^- produced as a by-product by the Krebs cycle, the roots of which may extend to the earliest days of intermediary metabolism [30,31]. It is noteworthy that archaeans lack class III ACs. Possibly because of their unusual metabolism, archaeans may have had less need to detect metabolic CO_2/HCO_3^- and hence lost type III ACs. Alternatively, type III ACs may have been derived by bacteria after their divergence from archaeans.

Genetic and genomic data strongly support the hypothesis that the proto-mitochondrion was an α-proteobacterium [32]. A consensus is also emerging that the host of the proto-mitochondrion was an archaean [33]. A parsimonious explanation for the observed distribution of class III ACs is that they were brought to eukaryotes as part of an intact signaling module via the mitochondrial endosymbiosis (Fig. 2). Subsequent to this endosymbiosis, eukaryotes and archaeans diverged, i.e., archaeans that acquired protomitochondria became eukaryotes

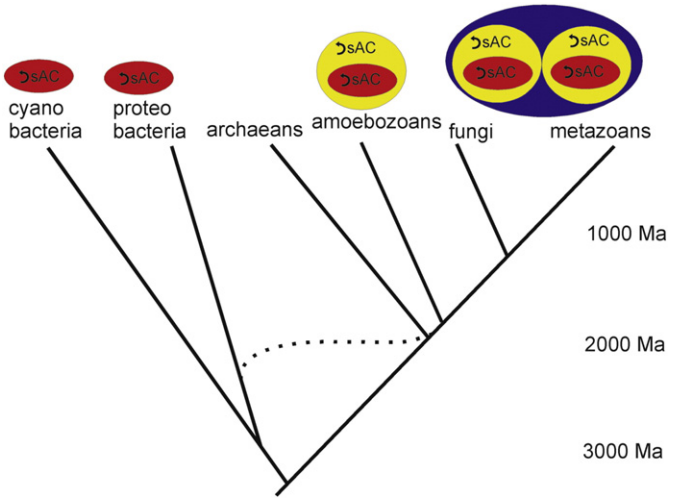

Fig. 2. Schemata depicting the evolution of the sAC signaling pathway. Solid lines indicate phylogenetic descent, while the dotted line shows the endosymbiotic event that led to the origin of eukaryotes. Timescale is approximate. The host of the proto-mitochondrion, an α-proteobacterium, is here depicted as an archaean, which diverged from other archaeans at the time of the endosymbiosis. In modern taxa, type III adenylyl cyclases, which include sAC, are found in bacteria, unicellular eukaryotes, and multicellular eukaryotes. The sAC signaling module may have been transferred intact to eukaryotes by the mitochondrial endosymbiosis. After transfer to the proto-nuclear genome, signaling with sAC was co-opted into signaling in unicellular eukaryotes and ultimately in multicellular eukaryotes.

[34]. One of the many consequences of this transition was that the proto-eukaryote inherited a bacterial metabolism and hence a bacterial system for regulating this metabolism, i.e., class III ACs.

Modern α-proteobacteria are diverse, both taxonomically [35] and metabolically, often exhibiting both photosynthesis and respiration [36]. Class III ACs are found in α-proteobacteria [20,21], but functional studies have focused on cyanobacteria, in which cAMP has been found to regulate respiration [37]. Metabolic CO_2/HCO_3^- may modulate this effect via sAC signaling [38]. While further functional studies are clearly required, a role for sAC in the regulation of respiration may also be found in α-proteobacteria. At least several of the extant α-proteobacteria exhibit respiratory chains and respiratory control strikingly similar to modern mitochondria [39,40]. A reasonable hypothesis is that sAC signaling functioned in proto-mitochondria much as it does in modern mitochondria.

A role for sAC in the regulation of mitochondrial function remains a relatively new and unexplored notion, with existing studies confined to mammalian systems [41–44]. Metabolic signals (e.g., CO_2/HCO_3^-) seem to be transduced by sAC and cAMP, but whether protein kinase A activation occurs remains an area of controversy. Here it will be assumed that the canonical sAC pathway is widely found in mitochondria. This assumption is not critical; however, it is a critical assumption that CO_2/HCO_3^-/sAC/cAMP modulates respiration in a wide diversity of eukaryotes. With the increasing discovery of sAC genes in metazoans in particular [45], additional opportunities for ground-breaking functional studies are possible.

The theoretical framework for evolutionary transitions, outlined above, suggests that levels-of-selection conflicts were inevitable in the origin of eukaryotes. In other words, selection acting on individual proto-mitochondria within a host cell favored different traits than selection acting on the group of proto-mitochondria within a single host cell. In order for the higher-level unit to emerge, mechanisms to mediate these conflicts necessarily had to evolve. The higher-level unit is not equivalent to the host cell. Rather, it is a community of prokaryotes including one host and potentially many proto-mitochondria. As pointed out above, mechanisms that mediate conflicts typically decrease variation among lower-level units or increase variation among higher-level units [12]. In the former case, selfish lower-level units are less likely to evolve. In the latter case, selection can remove higher-level units that function poorly because of lack of cooperation among lower-level units.

Cooperation can arise as a by-product of selection for metabolic homeostasis in individual proto-mitochondria [7]. State 3 proto-mitochondria efficiently convert substrate into ATP with moderate production of ROS. If substrate supply overmatches metabolic demand (as for instance in human diabetes, e.g. [46]), the levels of ATP relative to ADP become maximal, and the redox state of the electron carriers shifts in the direction of reduction (Fig. 1c). Under these state 4 conditions, ROS production increases dramatically, and such proto-mitochondria can damage themselves. From the perspective of proto-mitochondria, an oversupply of ATP is a liability. The adenine nucleotide translocator (ANT) was thus an evolutionary innovation to dispose of surplus ATP. ANT evolved due to selection on individual proto-mitochondria for metabolic homeostasis. ANT did not evolve to promote group-level cooperation, but it had that effect as a by-product. The distinction is important, since early in the endosymbiosis group-level selection (i.e., selection on the emerging proto-eukaryote) is generally conceded to have been weak relative to individual-level selection (i.e., selection on individual proto-mitochondria).

Nevertheless, with the invention of ANT, the proto-eukaryote began to emerge as an evolutionary unit [7]. ANT also contributed to signaling between the symbiont community and the cytosol. Signaling with sAC can be viewed in this context. As substrate was oxidized in proto-mitochondria, NADH, $FADH_2$, and carbon dioxide were produced (Fig. 1c). CO_2/HCO_3^- activated sAC and initiated the cAMP signaling that "primed" the electron transport chain for the arrival of NADH and $FADH_2$. In this way, proto-mitochondria avoided state 4 and maintained state 3. Signaling with sAC in proto-mitochondria could thus maintain metabolic homeostasis and constrain proto-mitochondrial variation [7].

If sAC signaling was a proto-mitochondrial trait, it could nevertheless quickly be transferred to the cytoplasm of the proto-eukaryote. Damaged proto-mitochondria would have released their DNA into the cytoplasm. Prior to the evolution of the nucleus, this DNA could quickly incorporate into the host genome. Recombination would associate proto-mitochondrial genes with host promoters. Proto-mitochondrial genes would then be expressed in the cytosol [47]. The sAC pathway could then be recruited into signaling roles in proto-eukaryotes very early in the endosymbiosis. Such traits may have provided a fitness advantage for the proto-eukaryotes that exhibited them.

Signaling with sAC was thus co-opted into signaling in unicellular eukaryotes and ultimately in multicellular eukaryotes (Fig. 2). Remarkably, in animals and fungi sAC signaling seems to have a role in independently derived transitions in multicellularity that occur during the life cycle [7]. Signaling with sAC and other class III ACs is involved in sperm development, at least in deuterostomes (e.g., echinoderms [48] and mammals [49]). Since features of sperm are derived in animals, use of class III ACs in sperm development is likely derived as well. Fungi use sAC signaling in the transition from unicellular to multinucleate, hyphal growth [50]. The latter characteristic is derived in fungi relative to other opisthokonts.

Perhaps the best known case of signaling with class III ACs in the unicellular-to-multicellular transition is found in the well-studied amoebozoan, *Dictyostelium discoideum*. This species has several adenylyl cyclases. The catalytic domains show that these are clearly typical eukaryotic class III ACs [25]. These ACs produce cAMP that is used in many aspects of the biology of *D. discoideum* [51]. Perhaps the most striking example occurs when some of the free-living amoebas become food-limited. These cells produce and secrete cAMP at regular intervals. The original pulse of cAMP triggers other cells to secrete cAMP, propagating the signal in a spiral and prompting movement of cells toward the source. Ultimately, the multicellular phase of the life cycle is initiated.

The use of sAC signaling in transitions in multicellularity has thus been independently derived in at least three groups of eukaryotes. This supports the hypothesis that mechanisms that mediated conflicts in the prokaryote-to-eukaryote transition may be co-opted to mediate conflicts in subsequent transitions to multicellularity. More general insight into the evolution of multicellularity may also be provided. Relative to animals and fungi, amoebozoans likely exhibit a less-derived form of multicellularity. Thus the initial steps in the evolution of multicellularity may generally have been connected to food supply, i.e., the advantages of greater size only outweighed the disadvantages when food was limiting. Since it is triggered by by-products of metabolism, CO_2/HCO_3^-, and by derivatives of the primary energy currency, ATP, sAC signaling is closely related to metabolism and metabolic state. Metabolic signaling via sAC may be a form of "honest signaling," in which the signal provides an accurate barometer of metabolic state [52]. Deceptive signaling can lead to conflict among lower-level units, but such conflict is less likely when honest signals are employed. In this way, having class III ACs may generally ease the transition to multicellularity.

There may be other instances where signaling with sAC mediates conflict. Consider a unicellular eukaryote with not only mitochondria but chloroplasts as well. The former are an intracellular source of metabolic CO_2/HCO_3^-, while the latter are a sink. In some environments (e.g., shade), production of CO_2/HCO_3^- by the source may exceed consumption by the sink. Via sAC, excess CO_2/HCO_3^- may then activate ciliary action and migration. Once a more favorable environment is attained, homeostasis is restored. Parallel dynamics may occur in coral bleaching, which is initiated by impaired photosynthesis in symbiotic dinoflagellates [53]. Corals contain sAC genes and exhibit high levels of cAMP production [54]. Symbiont migration, driven by ciliated coral cells lining the gastrovascular system, may thus result directly from impaired photosynthesis [55].

4. Other pathways

sAC is found in a wide diversity of organisms [20,21,25,27,45]. While further functional studies are required, this pattern of diversity allows relatively strong inference to be made concerning its evolution. This unfortunately is not so clearly the case for other important signaling pathways that display intriguing mitochondrial connections. By way of comparison, two of these (involving STAT3 and VEGF-B) are briefly reviewed here.

STAT3 is a member of the Signal Transducer and Activator of Transcription (STAT) family. After tyrosine phosphorylation, STAT3 acts as a transcription factor in the usual manner [56]. However, STAT3 can also be activated by serine phosphorylation, whereupon it localizes to mitochondria [57,58] and possibly functions as a component of complex I and II of the mitochondrial electron transport chain. Cells deficient in STAT3 exhibit a diminished capacity for oxidative phosphorylation [58]. The mitochondrial role for STAT3 may be connected to the abnormal mitochondrial metabolism that characterizes cancer cells. Indeed, mitochondrial STAT3 appears to contribute to Ras-dependent cellular transformation by altering the activity of complexes of the electron transport chain as well as somehow upregulating glycolysis [57].

In parallel to sAC signaling, a reasonable hypothesis is that the role of STAT3 in the mitochondrial electron transport chain was its ancestral function, while between-cell signaling was derived [59]. Testing this hypothesis, however, is challenging. JAK–STAT signaling diversified primarily in the vertebrate animals, and these have been the focus of most studies. Phylogenetic analysis of JAK–STAT pathway components [60] may be consistent with STAT3 representing the ancestral STAT function (e.g., Fig. 12 of [60]). A Stat-like protein similar to STAT3 has also been detected using immunochemical assays in bacteria [61]. Understanding the evolution of STAT3 clearly requires better characterization of the distribution and function of Stat-like proteins in bacteria and non-metazoan eukaryotes. Against the background of mechanisms generating gene diversity in eukaryotes (e.g., gene duplication and domain shuffling, [60,62]), it may not be possible to definitively resolve this history. Nevertheless, the idea of a proto-mitochondrial function mediating metabolism followed by co-option into a role in cell–cell signaling is intriguing particularly in the context of the example of sAC signaling outlined above.

Vascular endothelial growth factor (VEGF) proteins are major regulators of angiogenesis as well as other aspects of endothelial cell physiology in mammals. VEGF-B reveals a connection between the VEGF family of proteins and mitochondrial metabolism [63]. Subsequent to the appropriate metabolic signal, VEGF-B and a battery of mitochondrial genes are upregulated. The mitochondrial proteins thus produced are involved in the transport and β-oxidation of fatty acids. VEGF-B may thus be an important regulator of mitochondrial metabolism. Could this be an ancestral function? VEGF genes are part of a large family of platelet-derived growth factors (PDGF) that are conserved throughout the metazoans [64]. VEGF receptors are a type of receptor tyrosine kinase that diversified with the animals [65]. This suggests that the role of VEGF in angiogenesis is a derived feature of the metazoans. Cystine-knot proteins, which include VEGF/PDGF and many others, are widespread in eukaryotes. These proteins, however, diverge widely in sequence similarity and may be independently derived in different eukaryotic groups [66]. Some similarities between metazoan cystine-knot proteins and those of fungi and amoebozoans are nevertheless found [67], although interpretations of these similarities may differ.

Finding a VEGF protein with a mitochondrial function in animals may suggest that this is a derived function. A VEGF family protein may have been co-opted into a mitochondrial role to better co-ordinate mitochondrial function with angiogenesis. Alternatively, it may be that other VEGF/PDGF proteins have mitochondrial functions that have not yet been detected. In a broader sense, the role of modern VEGF-B may suggest the deep roots of cystine-knot signaling in eukaryotes. Cysteine-rich proteins may generally be expected to function in redox-regulated signaling [68]. A connection between such redox-regulated proteins and ancestral cystine-knot proteins may thus be expected.

More generally, a pathway need not have been present in α-proteobacteria to have mediated conflict in the prokaryote-to-eukaryote transition. Proteins may have been recruited from the host or the symbiont and new pathways derived to mediate conflicts. Plausibly, the original function of certain cysteine-rich proteins was to activate fatty acid transporter proteins in the proto-eukaryote. These proteins may have originated as symbiont proteins that connected proto-mitochondria to an external food supply. Alternatively, they may have originated as host proteins that regulated the availability of food for proto-mitochondria. By regulating proto-mitochondrial metabolic state, these cysteine-rich proteins could mediate conflicts. These proteins may have maintained this function in unicellular eukaryotes. Except for a few key residues, their amino acid sequences rapidly diverged in different lineages, resulting in an uncertain pattern of homology. With the origin of metazoans and possibly other multicellular eukaryotes, these proteins may have been recruited to new functions involving conflict mediation, e.g., angiogenesis (Fig. 3). Nevertheless, the complexities of gene diversification in eukaryotes may make this history very difficult to elucidate.

In any event, both STAT3 and VEGF-B are important regulators of mitochondrial metabolism. As suggested by the sAC example, such regulators may have mediated conflict in the prokaryote-to-eukaryote transition. The modern roles of STAT-family proteins (regulation of immune function) and VEGF-family proteins (angiogenesis) suggest a prominent role in the mediation of cell-cell conflict. While it remains unclear whether the history of either of these proteins can be reliably traced, further support for the perspective developed here may still be suggested. When eukaryotes become multicellular, these within-cell pathways of conflict mediation may then have been co-opted into between-cell pathways. The success of eukaryotes in mediating within-cell conflicts may have preordained their success in mediating between-cell conflicts and thus achieving multicellularity. Certainly, eukaryotes have achieved notable success as multicellular organisms [69]. The challenges that had to be overcome in forming a higher-level unit out of a community of energy-converting lower-level units [70] may have given eukaryotes a remarkable toolkit to overcome such conflicts in subsequent evolutionary transitions. Perhaps this is the evolutionary version of the familiar Nietzschean quote—what doesn't kill you makes you stronger, or at least more evolvable.

5. Discussion

Signaling pathways, like genes, are rarely created de novo. Rather, the existing material is typically co-opted into new and adaptive functions. During the major transitions in the history of life, there were both strong selection for new functions and a plenitude of material available to be co-opted. New signaling pathways may have been particularly likely to evolve during these transitions. Further, at least from an evolutionary perspective these transitions have repetitive features: conflicts among lower-level units had to be mediated in favor of the higher-level unit. It thus seems logical or at least possible that mechanisms of conflict mediation from earlier transitions (e.g., prokaryote to eukaryote) could be co-opted into similar functions in later transitions (e.g., the repeated evolution of multicellularity). Testing this hypothesis, however, is challenging. With constant extinctions, losses, and reversals, the history of many signaling pathways is as fragmentary as our knowledge of these pathways.

A recent study of the evolution of calcium signaling provides an instructive example [4]. Calcium signaling has many functions both between cells and within cells. Consequently, there are many protein components to this signaling system—pumps and exchangers, plasma membrane channels, organelle channels, buffers, and so on. Using available genome sequences of a diverse array of eukaryotes, Collins and

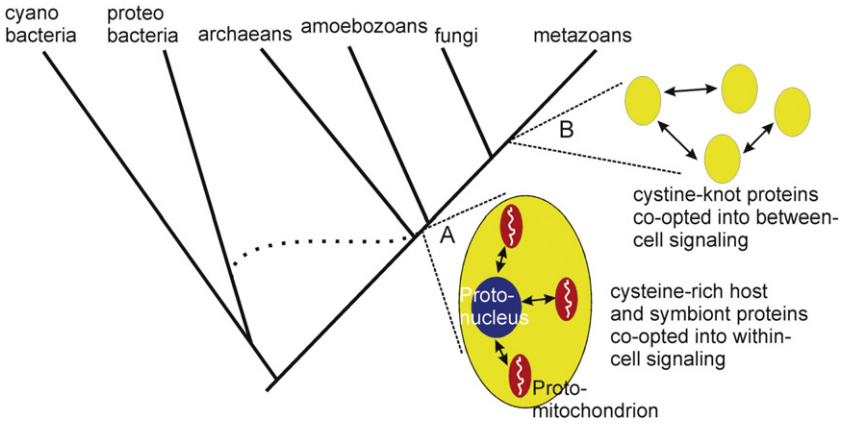

Fig. 3. Schemata depicting one scenario of the evolution of signaling pathways such as the VEGF-B pathway. Phylogeny is the same as Fig. 2. During the period of rapid evolution following the origin of eukaryotes, redox-sensitive, cysteine-rich proteins from both the host and symbiont may have been co-opted into new signaling pathways (A). These pathways may have maintained metabolic homeostasis and thus mediated conflict in the proto-eukaryote. As multicellular eukaryotes evolved, pathways mediating cell–cell conflict evolved (B). Pathways that involved cystine-knot proteins, including VEGF/PDGF, may have been co-opted from redox-sensitive pathways used to regulate proto-mitochondria. In some cases, features connected to mitochondrial metabolism may have been retained, as in VEGF-B.

Meyer [4] determine whether homologs to the human genes for these proteins exist in these various taxa and carry out a phylogenetic analysis of the resulting sequences. This analysis strongly supports the idea that the first eukaryotes utilized calcium signals and points to some interesting evolutionary patterns, e.g., Ca^{2+}-regulated phospholipase Cδ (PLCδ), which can produce IP_3 (inositol triphosphate), is more broadly distributed and possibly arose earlier than IP_3R (inositol triphosphate receptor). Appropriately, the authors caution that the ion selectivity and subcellular location of the identified homologs cannot be inferred from the sequence data. Repeated losses of seemly critical components of signaling pathways are also apparent. Further, functional evidence sometimes conflicts with the sequence data, e.g., some species that apparently lack IP_3R homologs nonetheless exhibit IP_3-mediated Ca^{2+} signaling.

As suggested by STAT3 and VEGFB, these data on the evolution of calcium signaling support the general notion that evolutionary analysis of events that occurred early in the history of eukaryotes are complicated by the evolutionary process itself [71,72]. Given the available data, there may be limits to tree-based inference [34]. Thus, if an organism lacks a particular protein, it still may carry out a particular signaling pathway using different means. At the same time, the presence of a particular protein does not necessarily mean that a particular signaling function is also carried out. Protein functions are lost by suppression or gene loss, and protein functions are gained by co-option. Meanwhile, taxa with intermediate character states can be lost to extinction.

For whatever reasons—ubiquity, necessity, chance—some pathways may provide an unusually complete record—the molecular Lagerstätten. Such pathways can provide a "proof of concept" that can be extended to more fragmentary remains. sAC signaling represents such an example. Its distribution and function has been well studied for some time [25]. Remarkably, signaling of type III ACs seems to be not only widely distributed but strikingly consistent. The same signaling module appears in bacteria and eukaryotes. In the latter, type III ACs are found in both mitochondrial and cytosolic compartments. At least some functions are shared, e.g., in the bacteria and mitochondria that have been studied, sAC signaling stimulates respiration [38,41]. The connections between sAC and critical metabolites (CO_2/HCO_3^-) explain both its ubiquity and conserved function. Type III ACs appear to be absent in archaeans. In part, this may indicate the unusual metabolism and limited characterization of archaeans. Nevertheless, both the apparent absence from archaeans and the similarity between mitochondrial and bacterial pathways support the hypothesis that this signaling pathway was acquired by eukaryotes from proto-mitochondria.

The same could perhaps be suggested for a number of other signaling pathways, including those involving STAT and VEGF proteins and calcium for that matter [6]. As suggested above, the evolutionary history of these signaling pathways is considerably less clear than that of sAC signaling. Yet in each case a possible connection to the mitochondrial endosymbiosis is suggested. Of course, the centrality of mitochondria to metabolism and metabolic signaling provides selective impetus for involvement of mitochondria in signaling. Evolution is never purely about function, however; history plays a role as well. Going forward, further characterization of rare, possibly vestigial, functions of major signaling pathways may provide invaluable insight. Examining signaling functions in obscure, yet phylogenetically relevant taxa may also be illuminating. Such additional data will better clarify the extent to which sAC may serve as a general model for the effects of endosymbiosis on signaling pathways.

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