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An antimicrobial origin of transit peptides accounts for early endosymbiotic events

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Primary endosymbiosis, which gave rise to mitochondria or chloroplasts, required successful targeting of a number of proteins from the host cytosol to the endosymbiotic organelles. A survey of studies published in separate fields of biological research over the past 40 years argues for an antimicrobial origin of targeting peptides. It is proposed that mitochondria and chloroplast derive from microbes that developed a resistance strategy to antimicrobial peptides that consisted in their rapid internalization and proteolytic disposal by microbial peptidases.

KEYWORDS

antimicrobial peptide, chloroplast, endosymbiosis, organelles, mitochondria, targeting peptide

1 | INTRODUCTION

Eukaryotic cells display several intracellular organelles but only mitochondria and plastids contain DNA. The identification and characterization of these genomes has been instrumental in ascribing their origin to ancestral-free living α-proteobacteria and cyanobacteria, respectively.[1–4] The endosymbiotic origin of chloroplast had been suggested as early as 1883 by Schimper[5] and by Mereschkowsky[6] then extended to the mitochondrion by Wallin.[7,8] Today, there is a consensus on the major traits of the postendosymbiotic events that led to contemporary eukaryotes. These encompass a considerable shrinkage of the ancestral eubacterial genomes due to the loss and/or transfer of the vast majority of their genes to the nuclear compartment of the postendosymbiotic cell, this being known as endosymbiotic gene transfer.[9] Despite this massive gene transfer, the function of many gene products has been preserved, thanks to the establishment of an active protein translocation process back from the cytosol to the endosymbiotic organelle.[10,11] The concerted reallocation of these genes to the nucleus and of their protein products in the opposite direction, allowed the organelles to keep the critical functions in bioenergetics of their bacterial progenitors.

Many studies have provided experimental support to postendosymbiotic intracellular gene transfer, showing that it still occurs at a significant rate.[12,13] The mechanism for import of proteins from the nucleocytosol back to the organelle compartment, that was obscure in the early 1980s, as will be detailed below, is now rather well understood (for recent reviews[14,15]) thanks to pioneering studies on mitochondria performed in the Schatz and Neupert laboratories[16,17] as well as by several seminal contributions on plastid import (reviewed in[18]). However, the process that led to the establishment of organelle translocons remains a matter of debate,[10,19–21] that very much relies on the original function that had those subunits of the import complexes, which are of prokaryotic nature.[22–24]

This commentary presents a tentative scenario for the early establishment of a successful organelle protein import machinery, which is based on an antimicrobial origin of the organelle-targeting peptides. Its presentation requires a short survey of various studies that were conducted over the past 30 years, on quite separate issues pertaining to organelle biology, protein import, peptide biology and mechanisms of cell defense against pathogens.

2 | ENDOSYMBIOSIS REFLECTS THE STABILIZATION OF A TRANSIENT INTERACTION BETWEEN A PREY, OR A PATHOGEN AND THE HOST

The physiological conditions that prevailed at the time when the host cell and the endosymbiont started their metabolic integration have been—and still are—a matter of debate.[21,25–27] The assumption of a mutual benefit,[28] even if serendipitously experienced, involves specific conditions that led to the persistent internalization of the prokaryote by the host cell. In that respect, it is now widely accepted that, among all α-proteobacteria branches, the mitochondrial ancestor

roots close to Rickettsiaceae that are obligate intracellular pathogens.[29] The plastid progenitor has been presented as a cyanobacterium that was a prey for a phagotrophic plantae ancestor (Reyes-Prieto and Weber 2007). Recently, Ball et al,[26] working on the metabolism of carbon storage in plants, suggested the plantae ancestor also had been attacked by a Chlamydiae pathogen that provided some unique genes for assimilation of photosynthetic carbon by the host. Interestingly, the latter scenario presents similarities with the mitochondrial endosymbiotic event. In this view, as suggested earlier by Margulis et al,[30] pathogen-attack processes would have been at the origin of the engulfment of the organelle progenitor that, owing to defense mechanisms from the host, would have evolved towards a symbiotic integration. Thus, endosymbiosis derives from transient interactions between a host and an organelle progenitor, were it be a prey or a pathogen. This holds for the first endosymbiotic event between an Archaea and a Rickettsia, that led to eukaryotism, and for the secondary event that led to formation of the algal/plant cell from a protist and a cyanobacterium—with or without concomitant Chlamydiae infection. Originally, the organelle progenitor was either destroyed by the host defense mechanism or the host was to collapse ultimately due to the pathogenic effect of its infection. Thus, at the core of the endosymbiotic theory stands the hypothesis that some unknown event led to retention of a prey, or disarmament of a pathogen, a prerequisite for the subsequent massive intracellular gene transfer to the host and massive protein import from the host cytosol back to the endosymbiont.

3 | THE EARLY 1980s: THE KEY DISCOVERY OF MITOCHONDRIAL AND CHLOROPLAST TARGETING PEPTIDES

A breakthrough in our understanding of endosymbiosis came in the late 70s with the recognition that proteins imported into chloroplasts and mitochondria were made as precursors in the cytosol before being processed to their mature size upon import.[31–33] Soon it was shown that an N-terminal presequence in the precursor protein bore all the information required for successful protein translocation.[34,35] About 5 decades of investigations disclosed many exceptions to this rule, that may hold for about 10%-30% of the organellar proteins (for reviews[36–38]). However, it remains that the main route for organelle protein import involves the specific recognition of the outer membrane of the organelle by a protein presequence that is removed and destroyed after import.[39,40] These presequences are hereafter mentioned as either mTP (mitochondrial targeting peptide) or cTP (chloroplast targeting peptide), for mitochondrial or chloroplast targeting peptide.

In the earliest studies from the 1980s, 2 main issues were rapidly raised: what were the sequence properties of targeting peptides that may explain their role in protein translocation across membranes and what were the molecular bases for their interaction with their target membrane?

Sequence analysis provided 2 important pieces of information with respect to the above issues. Targeting peptides are diverse in terms of primary sequence and length, from 50 to 70 residues for CTPs with an average value of about 55 residues, and from 20 to 70 residues for MTPs with an average value of about 45 residues, but their amino acid composition shows preserved physicochemical characteristics, with an enrichment in hydroxylated, hydrophobic and basic residues and a quasi-absence of acidic residues.[41,42] In a systematic comparison of all known MTPs and CTPs from *Arabidopsis thaliana*, Glaser and colleagues[43] showed their strikingly similar amino acid composition except for an additional N-terminal segment of approximately 16 residues in CTPs. Targeting peptides also showed domain organization and secondary features that suggested particular functions. In particular, besides the organization in subdomains for intrachloroplast sorting (for reviews see[44,45]), the most N-terminal part of MTPs display a positively charged domain with basic residues engaged in an amphiphilic helix[46] whereas the corresponding domain in CTPs is an unstructured hydroxylated rich sequence, which folds in an amphiphilic helix when exposed to an hydrophobic interface.[47,48] Because a plant cell houses the 2 types of organelles, the above differences should contribute to the differential sorting of proteins between chloroplasts and mitochondria. It is of note that algal cells such as Chlamydomonas display CTPs that more closely resemble MTPs, with an enrichment in basic residues and a clearly defined amphiphilic helix.[49] In conclusion, despite their diversity in length, sequence, domain organization and final destination within the organelle, targeting peptides share this ability to spontaneously form an amphiphilic α-helix when interacting with their target membrane.

Peptide amphiphilicity has been recognized as a key feature of the interaction with the lipid bilayer of the target membranes,[50,51] being responsible for the ability of mTPs and cTPs to penetrate in some depth into the membrane, most probably by reorganizing the polar head groups of lipids along the hydrophilic face of the amphiphilic helix.[52] Such evidence arose from structural studies of lipid reorganization upon exposure of liposomes to targeting peptides[52–57] as well as from functional analysis concluding to the bioenergetic uncoupling of organelle membranes when incubated with these peptides.[58,59] Although it has been suggested that targeting peptides per se could create channels that would allow their passenger proteins to proceed across the membrane bilayer,[59,60] most studies from the 1980s already considered that a receptor protein was required for successful protein translocation because a proteinase treatment of organelle membranes abolished protein translocation in an import assay.[61,62] Still, in this view as well, the ability of targeting peptides to reorganize lipid bilayers was considered as instrumental to the import process.[42]

4 | THE SHIFT IN FOCUS IN THE 1990s: THE IDENTIFICATION OF TRANSLOCATION MACHINERIES ACROSS THE MITOCHONDRIAL AND CHLOROPLAST MEMBRANES

The interest for the physicochemical properties of targeting sequences collapsed in the early 1990s with the growing evidence that there were genuine translocation complexes, or import pores, across the 2 mitochondrial membranes[16,63–65] and across the 2 envelop membranes of the chloroplast.[66,67] A wealth of studies

since have been devoted to the characterization of the mitochondrial and chloroplast translocons, that span the outer and inner envelop membranes of each organelle, the TOM/TIM (Translocon across outer mitochondrial membrane/inner mitochondrial membrane) and TOC/TIC (translocon across outer chloroplast envelop/inner chloroplast envelop complexes from mitochondria and chloroplast, and to related pores for specific import of subclasses of proteins (for recent reviews see[14,36,68,69]). It is of note that the identification of bona fide components of translocons is not completely settled yet, as illustrated recently by the disputed claim that YCF1, an essential chloroplast-encoded protein, is a major component of the TOC/TIC complex.[70–72]

The main features of the contemporary translocons argue for a dual evolutionary origin, with some subunits originating from the host, like TOC159, while others have unambiguously a prokaryotic origin, such as TOC75 and TIC20 (reviewed in[22,73,74]) or TOM40 and TIM23 (reviewed in[21]). Orthologs of the latter proteins in prokaryotes are involved in efflux processes, whether peptide or metabolite export, or act as permeases for metabolite translocation.[23,75] Thus, they probably were reformatted to contribute a protein influx after endosymbiosis.

5 | IN THE 1990s, THE DISCOVERY OF ANTIMICROBIAL PEPTIDES IN MULTICELLULAR ORGANISMS LED TO THE RECOGNITION OF AN UBIQUITOUS INNATE IMMUNE SYSTEM IN LIVING CELLS

The antibacterial properties of a number of compounds secreted by fungi and bacteria have been known for a very long time. These were the source of many antibiotics produced during the second part of the last century. However, that most—if not all—living cells produced antimicrobial peptides via the actual transcription and translation of a set of defense genes had been largely ignored until the late 1980s. With the elucidation of insect defense mechanisms that were able to kill pathogenic bacteria, arose the concept of innate immunity[76] that was supported by the identification of families of Ribosomal-Associated Antimicrobial Peptides (RAMP) that were not derived from modifications of pre-existing metabolites but actually produced by the cells as a primary defense mechanism of ubiquitous significance (for reviews see[77,78]). Indeed RAMPs were identified in multicellular organisms and microorganisms, whether prokaryotes or eukaryotes. Their existence in Archaea also has been documented, although not much is known yet on the diversity of defense mechanisms in Archaea (for a review[79]). RAMPs, composed of 10 to 50 residues can be classified according to their primary sequence, overall charge, secondary structure and mode of action.[80] The major class is that of cationic RAMPs, with a subclass enriched in proline/arginine, another 1 enriched in critical disulfide bridges, and a larger subclass characterized by its ability to form an amphipathic α-helix when exposed to a lipid bilayer.[81] The latter family of linear cationic α-helical peptides, hereafter referred to as Helical-Amphiphilic-RAMP-(HA-RAMP) is widespread among multicellular organisms—290 different peptides, less than 40aa-long, encompassing insect cecropins, and amphibian bombinins, dermaseptins, magainins and esculantins.[77] It is also represented in prokaryotes by some bacteriocins in gram positive bacteria (less than 60 aa-long) and cecropin-like peptides in gram negative bacteria.[4] HA-RAMPs are positively charged due to the presence of basic residues that contribute to the hydrophilic surface of their amphiphilic α-helical domain. The structural properties of HA-RAMPs were further investigated in relation to their antibacterial activity and shown to contribute an interaction, via the basic residues of the peptide, with the negatively charged headgroups of bacterial phospholipids, along with hydrophobic interactions between the uncharged surface of the amphiphilic helix and the fatty acid chains of the lipid bilayer (for a review[82]). Membrane permeabilization by HA-RAMPs is well documented[83,84] and can be accounted for by a variety of models, barrel-stave, carpet or toroidal models.[77,85]

6 | SIMILARITIES BETWEEN TPs AND HA-RAMPs

From the brief description above of TPs and HA-RAMPs, it is apparent that they are both characterized by their ability to fold in an amphiphilic α-helix when exposed to an hydrophilic/hydrophobic interface. TPs and HA-RAMPs indeed have similar secondary structure as determined using NMR (nuclear magnetic resonnance) studies[41,86] and their helices, being rich in cationic and/or hydroxylated residues, even display similarities in primary sequences in some instances, as for the cTP of Chlamydomonas RubisCO activase the helix of which reads c-VQLQA*RRVSR*TAV*R*-n and that of the frog HA-RAMP dermaseptin S4, which reads n-WKTLL*KKVLK*AAA*K*-c. All these similarities have drawn the attention of several authors who performed parallel studies of the ability of both types of peptides to disorganize lipid bilayers[82,87] or to display antibacterial activity, as reported for mTPs from yeast.[58] Given that the endosymbiotic theory entails a conflict between 2 microorganisms that ultimately evolved in a symbiotic survival, the possibility that targeting peptides and HA-RAMPs share a common ancestor is appealing.

7 | AN AMPHIPHILIC HA-RAMP-BASED SCENARIO FOR THE ESTABLISHMENT OF A SUSTAINABLE INTERACTION BETWEEN THE PREY/PATHOGEN AND THE HOST

It has long been considered that the way organelle-targeting peptides were designed was not an issue for successful endosymbiosis. This common thought probably arose after an early study from the Schatz laboratory showing that about 2.7% of randomly cloned peptides from *Escherichia coli*, when fused upstream of a mitochondrial protein deleted for its own targeting sequence, would support, to some extent, its import in the organelle.[88] Similar observations were reported upon mutagenesis of the mature N-terminus of a mitochondrial protein lacking its original targeting sequence.[89] The authors from these studies noted, however, that the rates of import were very low, an observation which can be better understood in light of

our present knowledge. Protein import in contemporary organelles involves interactions of distinct domains of the preprotein with membrane lipids, with various chaperones and with various subunits of the translocon machinery, which all contribute to its proper targeting.[90–92] It is likely that these combined interactions were not completely destroyed in the experiments mentioned above, thus allowing a basal protein import of low efficiency by a variety peptide sequences. However, such import assays in contemporary eukaryotic cells using randomly designed peptides[50] do not address the key question that should be raised to understand the early events in endosymbiosis: what could be the process that allowed matching a set of peptides from the host genome with a proteinaceous machinery from the endosymbiont to produce the efficient import process borne by the present targeting peptides and organelle translocons?

The ubiquitous antimicrobial defense system operating in living cells was not known at the time when the organelle targeting sequences were first identified. To account for the early events which led to endosymbiosis, the author suggests that ribosomal antimicrobial peptides were likely to be produced by the host cell upon exposure to the endosymbiont progenitor. This would have occurred within archea when invaded by those pathogens that led to endosymbiosis of the mitochondrial progenitor, as well as within the phagotrophic protist when it internalized its prey together with/or without some pathogen, leading to endosymbiosis of the plastid progenitor. These defense mechanisms should have allowed archea to resist pathogen attacks and later on, protists to survive and/or to feed happily on their cyanobacterial prey.

The author suggests here an evolutionary relationship between HA-RAMPs and TPs that allows one to consider a 3-stage scenario describing early events in the endosymbiotic process (Figure 1).

7.1 | Stage 1: intricate life styles of the host and organelle progenitors allowed lateral DNA transfer

The pre-endosymbiotic interaction between the host cell and the organelle progenitor, which resulted in the successful killing of the pathogens and/or preys, should have elicited a substantial rate of lateral/horizontal gene transfer to the host cell. There is overwhelming evidence indeed for such DNA transfer within the prokaryotic world as well as between phagotrophic protists and their preys and more generally in contemporary host/pathogen systems.[93] This intricate life style is a prerequisite for the success of the next stages leading to endosymbiosis.

7.2 | Stage 2: microbial progenitors of mitochondria and chloroplasts developed resistance strategies to HA-RAMPs

A number of studies reported loss in efficiency of RAMPs in protecting a host cell from bacterial attacks (for reviews[94–96]). This was ascribed to the development of a various resistance mechanisms against different sets of RAMPS, ranging from bacterial membrane remodeling—allowing decreased electrostatic interactions with RAMPs due to an alteration of the membrane surface charge density—to RAMP proteolytic destruction by an exported bacterial protease or RAMP extrusion by an efficient efflux system born by ABC transporters once RAMPs sneaked into the microbe. Another mechanism of resistance involves RAMP trapping by establishment of a new import process and their subsequent destruction by proteolysis. This import is performed by a particular type of ABC transporters,

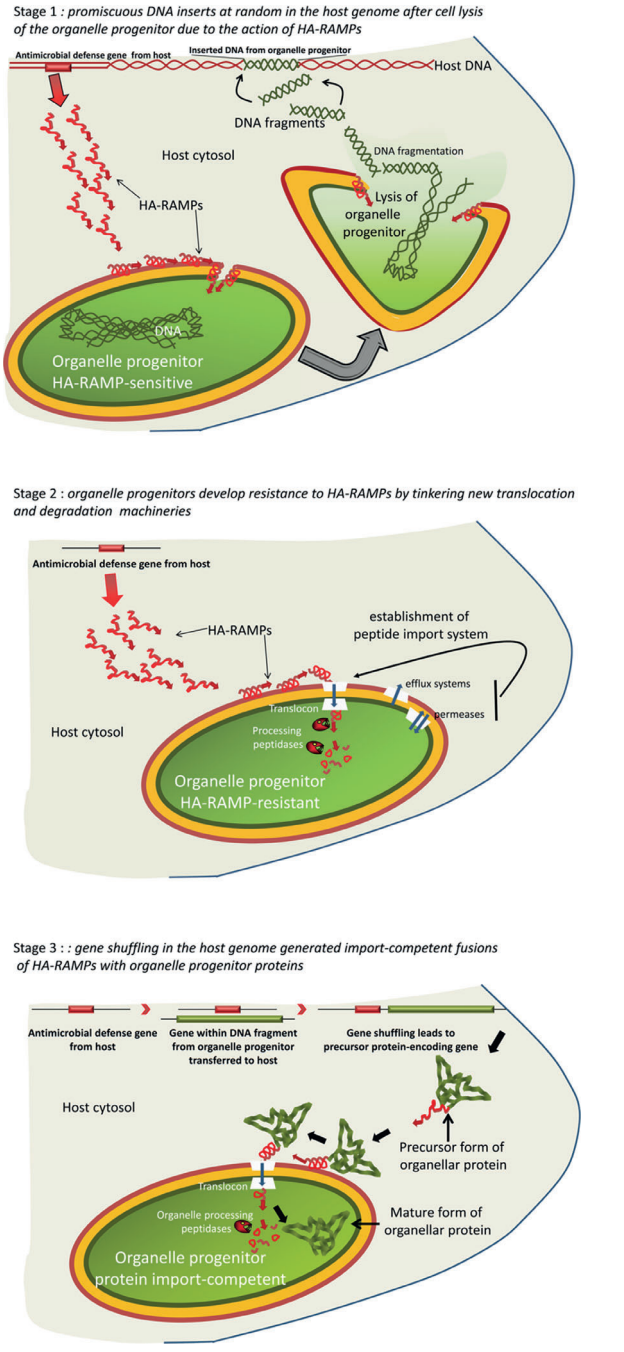

FIGURE 1 Early events in endosymbiosis when a prokaryote became the organelle progenitor. At stage 1, promiscuous DNA from the progenitor, a pathogen (mitochondria) or a prey (chloroplasts) inserts in the host genome due to cell lysis under the action of the host Ribosomal-Associated Antimicrobial Peptides (RAMPs). At stage 2, the progenitor has become resistant to the host RAMPs through their translocation and rapid destruction. The progenitor is now retained in the host. At stage 3, gene shuffling in the host genome allows back-import of progenitor gene products from the host cytosol to the progenitor. The way is now open to the evolution of the progenitor towards contemporary organelles.

the Bce-AB transporters, (reviewed in[97]). These have been identified for instance in the intracellular pathogen *Salmonella typhimurium*,[98] in *Bacillus subtilis*,[99] and *Staphylococcus aureus*.[100]

The latter resistance strategy, displays features that strikingly resemble those of the import systems in contemporary organelles. It may have proved a most efficient resistance system on the way towards endosymbiosis. Successful translocation of HA-RAMPs in the cytoplasm of the organelle progenitor required the recruitment of bacterial proteins originally contributing to molecular efflux or acting as permeases, as supported by the presence, in contemporary organelle translocons, of subunits showing sequence similarities to such prokaryotic proteins. Here, the functional principle of peptide translocation, rather than the conservation of protein sequences, argues for the evolutionary relation between HA-RAMPs and targeting peptides. As to the degradation of HA-RAMPs by dedicated proteases, indeed mitochondria and chloroplasts do house a very efficient proteolytic machinery to rapidly dispose of the targeting peptides once their translocation is completed. Translocated mTPs and cTPs are immediately recognized and processed by an endo-peptidase, the mitochondrial processing peptidase (MPP) or the stromal processing peptidase (SPP) which are evolutionary related. These peptidases release the mature protein in the matrix/stroma of the organelle, and the released transit peptide is rapidly degraded by dedicated peptidases, such as PreP and OOP in *A. thaliana* (for a review[40]).

7.3 | Stage 3: gene shuffling in the host-genome generated import-competent fusions of HA-RAMPs with organelle progenitor proteins

At the third and last stage of this scenario, the endosymbiont has developed the means to be retained in the host cell by inactivating its defense system through specific translocation and proteolytic machineries that allow it to import and destroy the HA-RAMPs delivered by the host. As mentioned earlier in stage 1, the resistance mechanism at stage 2 developed in a context where various DNA fragments of the endosymbiont progenitor already were randomly inserted into the host genome, because of the intertwined lives of the host and its bacterial prey or pathogen. Several bacterial coding sequences thus may have fused with upstream host genes encoding HA-RAMP, either upon random insertion or after gene shuffling, resulting in the expression of chimera containing genetic information from the organelle progenitor together with targeting information directed against the very same organelle progenitor. Such chimera, expressed in similar conditions as the other HA-RAMPs by the host expression machinery, were thus equipped with the recognition motives for being imported through the new translocon that provided bacterial resistance to HA-RAMPs. The proteolytic system inside the endosymbiont established for processing and degradation of the HA-RAMP peptides, thus restored the original bacterial protein sequence inside the organelle progenitor. The antimicrobial peptide had converted into a genuine targeting peptide.

Now that had been set up a new mechanism for protein import back to the organelle to which these protein-encoding genes once belonged, the traffic was open for further successful reallocation of genetic information between the 2 intracellular compartments. It should have allowed a high rate of evolution towards massive gene translocation to the host because of the presence of a molecular equipment for protein import, dedicated to successful functional complementation at the protein level of any gene loss from the organelle progenitor. After establishment of these proper molecular machines for successful metabolic integration, the conflict between 2 microorganisms could be converted into genuine endosymbiosis. The new energy resources brought along by the respiratory and photosynthetic processes from the endosymbiotic progenitors could be harnessed by the host to its greatest selective advantage.

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