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Review

Rhomboid proteases in mitochondria and plastids: Keeping organelles in shape ☆

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ABSTRACT

Rhomboids constitute the most widespread and conserved family of intramembrane cleaving proteases. They are key regulators of critical cellular processes in bacteria and animals, and are poised to play an equally important role also in plants. Among eukaryotes, a distinct subfamily of rhomboids, prototyped by the mammalian mitochondrial protein Parl, ensures the maintenance of the structural and functional integrity of mitochondria and plastids. Here, we discuss the studies that in the past decade have unveiled the role, regulation, and structure of this unique group of rhomboid proteases. This article is part of a Special Issue entitled: Protein Import and Quality Control in Mitochondria and Plastids.

1. Introduction

Proteases are enzymes that catalyze the hydrolysis of the peptide bond that links amino acids together in a protein; they participate in proteins catabolism and act as regulators of nearly every biological process [1]. Proteases are grouped in different catalytic types, depending on the catalytic amino acid residue used to conduct the nucleophilic attack on the peptide-bond of the substrate: aspartic, cysteine, glutamic, metallo (His), asparagine, threonine, and serine proteases. Due to the requirement of water for the proteolytic reaction, proteases were generally thought to hydrolyze their substrates in an aqueous environment. However, in 1997 Rawson and colleagues reported the discovery of site-2 protease, the first kind of protease capable of executing hydrolysis within the water-excluding environment of the phospholipid bilayer, which composes most of the biological membranes [2]. Such unique type of proteases are termed intramembrane-cleaving proteases, or I-CLiPs, and are structurally characterized by having the catalytic site residues projecting in a cavity that is buried within the lipid bilayer of the membrane in which the I-CliP is embedded [3].

To date, most of the I-CliP substrates appear to be membrane proteins; their cleavage typically occurs at or near the transmembrane helix (TMH) that anchors the substrate to the lipid bilayer [4,5]. Thus, their proteolysis serves to either complete protein maturation, or to release membrane-tethered domains, which are typically used for signaling [6]. Over the past decade, these released moieties have been shown to engage in intra- and inter-cellular communication, mainly by acting as transcription factors or ligands of signaling factors in a process that is termed regulated intramembrane proteolysis [7,8].

There are four known families of I-CliPs: site-2 protease, first discovered as a regulator of cholesterol biosynthesis [2,9]; presenilins/gamma secretase, implicated in Notch signaling [10] and Alzheimer's disease [11,12]; signal peptide peptidase [13,14]; and the rhomboids [15].

2. Rhomboids genes

The Rhomboid gene was first discovered in Drosophila. Fly developmental studies classified it under the Spitz group of genes, which includes *Spitz*, *Star*, *Pointed*, *Rhomboid*, *Single-minded*, and *Sichel*: mutations in any of these cause similar pattern alterations in ventral ectodermal derivatives of the fly embryo [16]. Because the names of these genes described the phenotype of the mutant larval cuticle, the mis-shaped, rhombus-like head skeleton of the mutant embryo earned to the *Rhomboid-1* gene its name.

Rhomboid genes are present in most of the sequenced bacterial, archaeal and eukaryotic genomes [17]. Most of the bacterial and archaeal genomes have a single rhomboid gene. In contrast, eukaryotes show expansion of the rhomboid family, with 2 members in yeast, 7 in Drosophila, 5 in humans and as many as 13 in *Arabidopsis t.* [18]. Phylogenetic analysis revealed that eukaryotic rhomboids are split between two

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major subfamilies, which are positioned in the midst of different prokaryotic branches [17]. The first subfamily was designated the RHO subfamily [17]; its prototype is the Drosophila developmental regulator Rho-1 [15,19–21]. The second eukaryotic subfamily was designated the PARL subfamily, and its prototype is the mammalian Parl protein which encodes for a mitochondrial inner membrane protein (discussed below) [17]. Whereas in plants the PARL family is considerably more represented, in animals there has been a selective expansion only of RHO family members; indeed, animal genomes typically contain only one PARL gene [17]. What evolutionary constraints have led, in animals, to the selective expansion of the RHO subfamily and, possibly, to the selected elimination of additional members of the PARL subfamily, remain unknown. Instead, the remarkable expansion of the PARL subfamily in plants appears to be linked to the presence of plastids: major plant- and algae-specific self-replicating organelles that are the site of photosynthetic fixation of CO_2 and are responsible for the exclusive manufacture and storage of starch and lipids. Although plastids greatly differ by their pigmentation, function, and ultrastructure, they are thought to have originated from endosymbiotic cyanobacteria [22]: such symbiosis parallels the event that lead to the acquisition of mitochondria in eukaryotes, which required the acquisition of a PARL family member to control inner membrane remodeling [18]. Thus, the expansion of the PARL family in plants could reflect the need to coordinate the remodeling and organization of the chloroplast thylakoid membrane, whose structure and dynamics is more complex than that of the mitochondrial *cristae*.

Phylogenetic analysis also identified a subgroup of rhomboid genes that express proteins lacking the catalytic residues: pseudoproteases [17]. These genes, which are present in all sequenced metazoans and are present only in metazoans, are termed iRhoms, for inactive rhomboids [23]. Unlike most sequenced metazoans, Drosophila has only a single iRhom gene, *rhomboid-5*. The high degree of conservation of iRhoms genes indicates that these pseudoproteases have been under evolutionary selection pressure, which in turn indicates that they possess a relevant biological function. Recent studies have indeed shown a critical role of iRhoms in regulating growth factor signaling through endoplasmic reticulum-associated protein degradation (ERAD) [24], and in the innate immunity response [25,26]. Derlin-1, a prominent component of the ERAD pathway [27], and iRhom2 [25,26] are the most recently characterized members of the iRhom group of pseudoproteases in mammals.

3. Rhomboids evolutionary origin

Phylogenetic analysis indicates that rhomboid family emerged in some bacterial lineage and widely disseminated by horizontal gene transfer (HGT) [17]. Both archaea and eukaryotes seem to have acquired rhomboids on several independent occasions. In particular, at least two HGT events seem to have contributed to the origin of eukaryotic rhomboids, one of them yielding the RHO subfamily and the other one the PARL subfamily, with a possible additional HGT in plants [17].

4. Rhomboids structure

Rhomboids are intramembrane-cleaving serine proteases. They are among the most conserved family of polytopic membrane proteins known to date and the most ancient type of I-CliP [17]. Prokaryotic and eukaryotic rhomboid proteins share only 10–15% of sequence identity in their catalytic domain (Fig. 1A); this is not surprising since polytopic transmembrane proteins are, in general, not strongly conserved. Rhomboid proteases from all kingdoms of life share the fact of having the catalytic domain composed by six TMHs [17]. Phenomenal work in protein crystallography has determined the structure of this domain in bacterial rhomboids: the overall shape is a compact helical bundle comprised of six TMHs and a lateral protrusion that connects TMH-1 and -2 [28–33]. Nearly the entire rhomboid domain appears to be immersed in the phospholipid bilayer; the lateral protrusion is only partially immersed in the membrane and might, therefore, serve to properly position the protease [33–35]. The universally conserved catalytic Ser residue, on TMH-4, lies submerged ~10 Å from the presumed plane of the membrane, making the site of catalysis intramembrane.

The structures of the bacterial rhomboids have revealed that the catalytic Ser and His residues in TMH-4 and TMH-6, respectively, are spatially arranged in a manner akin to classical serine proteases. In dyad-based serine proteases, the serine–histidine pair act together to generate a nucleophile of sufficient power to attack the carbonyl group of a peptide bond [36]. However, several serine proteases use a triad-based mechanism of catalysis [37]; here, a third catalytic residue, typically Asp or Asn, plays an important, but not essential, role in stabilizing the tetrahedral intermediate and the transition state leading to it. The structures of the bacterial rhomboids have unequivocally shown the existence of a catalytic dyad; indeed, a third conserved Asn residue, which was suspected to act as a third catalytic residue, is too remote and geometrically not in position to contact the catalytic His and form a triad [28–33]. However, recent studies form our laboratory have shown that eukaryotic members of the PARL family of mitochondrial rhomboid proteases, but not of the RHO family, could use a strongly conserved Asp residue on TMH-5 (Fig. 1D) in a triad-based mechanism of catalysis (Fig. 1C) [38]. The reason why a mitochondrial rhomboid would require a catalytic triad to properly work could be linked to the unique biophysical environment of the organelle. Bacterial rhomboid activity is highly sensitive to the phospholipid composition of the membrane in which they are embedded. Accordingly, the unique lipid composition of the inner mitochondrial membrane, along with the large electrochemical and pH gradient present inside the organelle, might have applied a selective evolutionary pressure that, ultimately, generated a form of the rhomboid protease with a more efficient mechanism of catalysis.

Unlike bacterial rhomboids, eukaryotic members of the RHO and PARL families possess seven TMHs; of these, 6 TMHs constitute the catalytic rhomboid domain. However, major structural differences differentiate these two types of rhomboid proteases. RHO family members, which are localized in the Golgi and plasma membrane, have the 7th TMH added at the carboxy-terminus of the 6-TMH rhomboid core; this topology is referred to as the "6 + 1" structure. Instead, the domain architecture of the rhomboids of the PARL subfamily, which in animals are exclusively found in the mitochondrion and in plants are likely localized in the membranes of the plastids, is characterized by having the 7th TMH added to the amino-terminus of the 6-TMH rhomboid domain; this topology constitutes the "1 + 6" structure [17,18]. The function of the seventh TMH, termed TMH-A and TMH-B in PARL and RHO family members, respectively (Fig. 2A), has not been established for any member of the PARL or RHO family. However, it may play a role in determining the correct topology and orientation of the protein in the membrane, in substrates gating or, in PARL family members, to orient TMH-5 in a way that allows the protease to use a catalytic triad [18,38] (Fig. 1C).

Structure–function studies of the mammalian Parl rhomboid domain shows a remarkable structural conservation of this fold with that of the bacterial GlpG protein (Fig. 1B). However, differences seem to exist. As mentioned above, our studies suggest that an Asp residue on the TMH-5 of Parl (D_{319}; Fig. 1D), which is not present in bacterial and RHO rhomboids, could play a critical structural and catalytic role. Indeed, replacing D_{319} with Asn, Glu, Ala, or Leu profoundly disrupted Parl expression and activity. In bacteria, TMH-5 is a highly dynamic helix [30,34]: a similar property in Parl could allow D_{319} to be positioned in close proximity, and on the same plane, of the catalytic H_{335} residue (Fig. 1C), thereby conferring to Parl a triad-based mechanism of catalysis. This is a likely possibility: mutations designed to assess whether TMH-5 may rotate to bring D_{319} into the active site (Fig. 1C) support indeed this scenario [38].