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Novel insights into the mitochondrial permeability transition

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Alavian and colleagues recently provided further evidence in support of the notion that the c subunit of the mitochondrial F_1F_O ATP synthase constitutes the long-sought pore-forming unit of the supramolecular complex responsible for the so-called 'mitochondrial permeability transition' (MPT). Besides shedding new light on the molecular mechanisms that underlie the MPT, these findings corroborate the notion that several components of the cell death machinery, including cytochrome c and the F_1F_O ATP synthase, mediate critical metabolic activities.

The term 'mitochondrial permeability transition' (MPT) is generally employed to indicate an abrupt increase of the permeability of the inner mitochondrial membrane (IMM) to small solutes. This results not only in the immediate dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$), and hence in the arrest of mitochondrial ATP synthesis, but also in a profound ionic imbalance that provokes the osmotic breakdown of the organelle.[1] Accordingly, widespread MPT marks the point-of-no-return of several instances of regulated cell death (RCD), often (but not only) manifesting with biochemical and morphological correlates of necrosis.[2-4] The MPT is caused by the opening of a relatively unselective multicomponent pore assembled at the junctions between the inner and outer mitochondrial membrane, the so-called 'permeability transition pore complex' (PTPC).[5,6] The pharmacological profile of the PTPC has been intensively investigated throughout the past 2 decades.[1,7] Nonetheless, its precise molecular composition, and in particular the identity of the pore-forming unit of the complex, remained a matter of debate until recently. Indeed, while a large number of studies pointed to various mitochondrial and cytosolic proteins as to structural or regulatory PTPC components, robust genetic evidence in support of these conclusions was lacking.[8-10] As a standalone exception, peptidylprolyl isomerase F (PPIF), a protein of the mitochondrial matrix best known as cyclophilin D (CYPD), has been ascribed a key and non-redundant regulatory role in the MPT as early as in 2005.[11-14]

Based on several lines of circumstantial evidence, including pharmacological profile and interacting partners,[7,15-17] we were among the first to hypothesize that the ATP synthasome, the multicomponent system that harnesses the electrochemical gradient generated across the IMM by the mitochondrial respiratory chain to produce ATP, is directly involved in the MPT.[18-20] In particular, we were the first to demonstrate that the c subunit of F_O (the IMM-embedded domain of the F_1F_O ATP synthase) is required for MPT, mitochondrial fragmentation and cell death as induced by mitochondrial Ca^{2+} overload and oxidative stress (two prototypic MPT inducers).[18] Our observations were confirmed a few months later by an independent study, proving that isolated c subunits form pores in artificial membranes and that these pores are sensitive to established MPT regulators, including Ca^{2+}.[21] In spite of these findings, however, the possibility that c-rings (i.e., the ring-shaped oligomers of c subunits that – together with other proteins – make up

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Abbreviations: AIFM1, apoptosis-inducing factor; mitochondrion-associated, 1; ATP5G, ATP synthase, H^+ transporting, mitochondrial F_O complex, subunit C; CsA, cyclosporin A; CYPD cyclophilin D; CYTC holocytochrome c; $\Delta\psi_m$; mitochondrial transmembrane potential; IMM, inner mitochondrial membrane; MCU, mitochondrial calcium uniporter; MPT, mitochondrial permeability transition; PPIF, peptidylprolyl isomerase F; PTPC, permeability transition pore complex; RCD, regulated cell death; SMV, submitochondrial vesicle.

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the F_O domain) would be available to generate MPT-like currents *in cellula* remained to be addressed.

The group of Elizabeth Jonas recently shed new light on the essential role played by the c subunit in MPT. By using reconstituted liposomes as well as purified mitochondria and submitochondrial vesicles (SMVs), Alavian and colleagues confirmed that c subunits can form multiconductance pores exhibiting a pharmacological profile that is similar to, but not perfectly overlapping with, that of the PTPC. On the one hand, the channel activity of highly purified c subunits reconstituted in liposomes as well as of SMVs exposed to urea (which denatures and removes extra-IMM proteins, including the F_1 domain) was shown to be insensitive to both Ca^{2+} ions and to cyclosporin A (CsA), a prototypical CYPD inhibitor, contrary to that of untreated SMVs and whole mitochondria. On the other hand, c subunit-containing liposomes and urea-treated SMVs displayed a channel activity that could still be inhibited by ATP, although with a reduced efficacy.[22] Taken together, these observations indicate that Ca^{2+} ions and CsA modulate the MPT by acting solely on extra-IMM components of the PTPC. Conversely, at least part of the MPT-inhibitory activity of ATP appears to reflects its ability to directly bind c-rings.

By means of the bipartite tetracysteine display method, Alavian *et al.* also demonstrated that CsA maintains c subunits close together in the IMM, as it protects human embryonic kidney HEK293 cells from the MPT-inducing effects of ionomycin (a ionophore that promotes mitochondrial Ca^{2+} accumulation). Conversely, control cells were shown to respond to ionomycin with a decrease in the relative proximity of c subunits. These data suggest that the MPT occurs *in cellula* along with a structural rearrangement of c-rings. Accordingly, the depletion of c subunits with short hairpin RNAs (shRNAs) specific for ATP synthase, H^+ transporting, mitochondrial F_O complex, subunit C1 (ATP5G1) or ATP5G3 (two of the three human isoforms of the c subunit) was found to resemble CsA in its ability to protect glycolytic cancer cells against MPT, $\Delta\psi_m$ dissipation and death as induced by ionomycin. Similar results were obtained with neurons maintained in normal medium and exposed to MPT-inducing agents such as H_2O_2 or glutamate. Conversely, the stable knockdown of ATP5G1 or ATP5G3 had a negative impact on the survival of HEK293 cells cultured in glucose-free, galactose-containing medium (which forces oxidative phosphorylation), an effect that could be rescued by means of a non-interferable c subunit-coding construct. Of note, a plasmid encoding a c subunit variant that contains 4 glycine-to-valine substitutions (increasing the steric hindrance of the protein and the conductance of c-rings) not only was unable to rescue the survival of HEK293 cells depleted of endogenous c subunits and maintained in these conditions, but also killed *per se* neurons cultured in normal medium in a CsA-insensitive manner.[22] These data suggest that alterations in the ability of c subunits to pack tightly influence the efficacy of mitochondrial ATP synthesis as well as the propensity of c-rings to form unselective pores across the IMM in response to MPT-promoting stimuli.

Finally, Alavian and colleagues reported that purified β subunits (which are part of the F_1 domain of the ATP synthase) inhibit the channel activity of purified c subunits reconstituted in liposomes. Taken together with the results obtained with urea-deprived SMVs, this observation suggests that the MPT may occur along with a physical dissociation between F_1 and c-rings. Indeed, c subunits were released from the F_OF_1 ATP synthase in the presence of Ca^{2+} ions, a process that could be blocked by CsA as well by the absence of CYPD (*i.e.*, in mitochondria isolated from *Ppif*$^{-/-}$ mice).[22] These findings are compatible with a model proposing that the CYPD-dependent binding of Ca^{2+} ions to the F_1 domain of ATP synthase displaces it from c-rings, hence initiating the MPT. Various components of the ATP synthasome, including solute carrier family 25, member 4 (SLC25A4), best known as adenine nucleotide translocase 1 and SLC25A3, best known as mitochondrial phosphate carrier, have been shown to alter the propensity of cells to undergo MPT-driven RCD,[23,24] but the underlying molecular mechanisms remain to be elucidated (**Figure 1**).

In conclusion, the report by Alavian and collaborators provide novel insights into the molecular mechanisms that underlie MPT-driven RCD. Several issues, however, remain unresolved. First, it will be interesting to precisely determine how c-rings form high conductance pores. Indeed, the flow of H^+ ions that powers ATP synthesis does not normally occur through the center of c-rings, which is highly hydrophobic, but at the interface between c-rings, other F_O subunits and the IMM.[25] Does the flow of small solutes that underpin the MPT occurs via the same route? Second, it will be important to understand how the association between F_O and F_1 ATP synthase domains is influenced by known regulators of the MPT, including metabolites, chemicals and PTPC interactors. Several members of the Bcl-2 protein family, including BCL2-like 1 (BCL2L1, best known as BCL-X_L) and BCL2-associated X protein (BAX) have been shown to interact with components of the ATP synthasome, hence regulating their functions.[17,24] Is the ability of these proteins to inhibit or promote MPT a direct consequence of such binding or does it reflect alterations in the local concentrations of ATP and ADP (both which are known MPT regulators)? Third, it will be crucial to characterize in detail the role of CYPD in the dissociation of F_1 domains from c-rings and the consequent initiation of MPT. As mentioned above, CYPD currently represents the only PTPC component firmly established by genetic data, but does not constitute its pore-forming unit. Mitochondria lacking CYPD require markedly increased amounts of Ca^{2+} ions to undergo the MPT, but are not completely resistant to the process.[12] Still, CYPD-deficient cells and mice are protected against a wide panel of MPT-triggering insults.[11,13,14] *Mcu*$^{-/-}$ mitochondria, which are unable to rapidly take up cytosolic Ca^{2+} as they lack the mitochondrial calcium uniporter (MCU), also do not undergo the MPT in response to standard Ca^{2+} levels (500 μM). *Mcu*$^{-/-}$ cells and animals, however, are as sensitive to MPT inducers as their

wild-type counterparts, yet fail to respond to CsA.[26] How can these observations be reconciled into a single MPT model? Further experiments are required to clarify these points.

Irrespective of these incognita, the data by Alavian and colleagues lend further support to the notion that several factors involved in the initiation or execution of RCD exert key metabolic or bioenergetic functions.[27,28] To mention two notable examples, this applies to holocytochrome c (CYTC) and apoptosis-inducing factor, mitochondrion-associated, 1 (AIFM1). Besides driving the assembly of the caspase-activating platform known as apoptosome upon mitochondrial outer membrane permeabilization (MOMP), CYTC shuttles electrons between respiratory complex III and IV, hence exerting a non-redundant vital function. Along similar lines, AIFM1 not only operates as a caspase-independent cell death executioner as it translocates to the nucleus upon MOMP or MPT and mediates large-scale DNA degradation, but also is required for the stability and function of respiratory complex I. Accordingly, both *Cyc1*$^{-/-}$ mice (lacking the protein moiety of CYTC) and *Aifm1*$^{-/-}$ mice do not survive through adulthood.[29,30] This has profound implications for cell death research, *de facto* precluding the generation of appropriate genetic models. In some instances, such an issue can be (at least partially) circumvented by the establishment of tissue-specific knock-out animals.[31] The gold standard approach to this problem would rely on the generation of mutant proteins that are unable to perform lethal functions but preserve their bioenergetic activities and homozygous knock-in mice. Such a strategy has been successfully undertaken by introducing a K72A substitution in the Cyc1-coding sequence, resulting in a CYTC variant that exerts normal respiratory functions but cannot bind apoptotic peptidase-activating factor 1 (APAF1) and hence cannot activate the caspase cascade.[32] Unfortunately, not all RCD-relevant

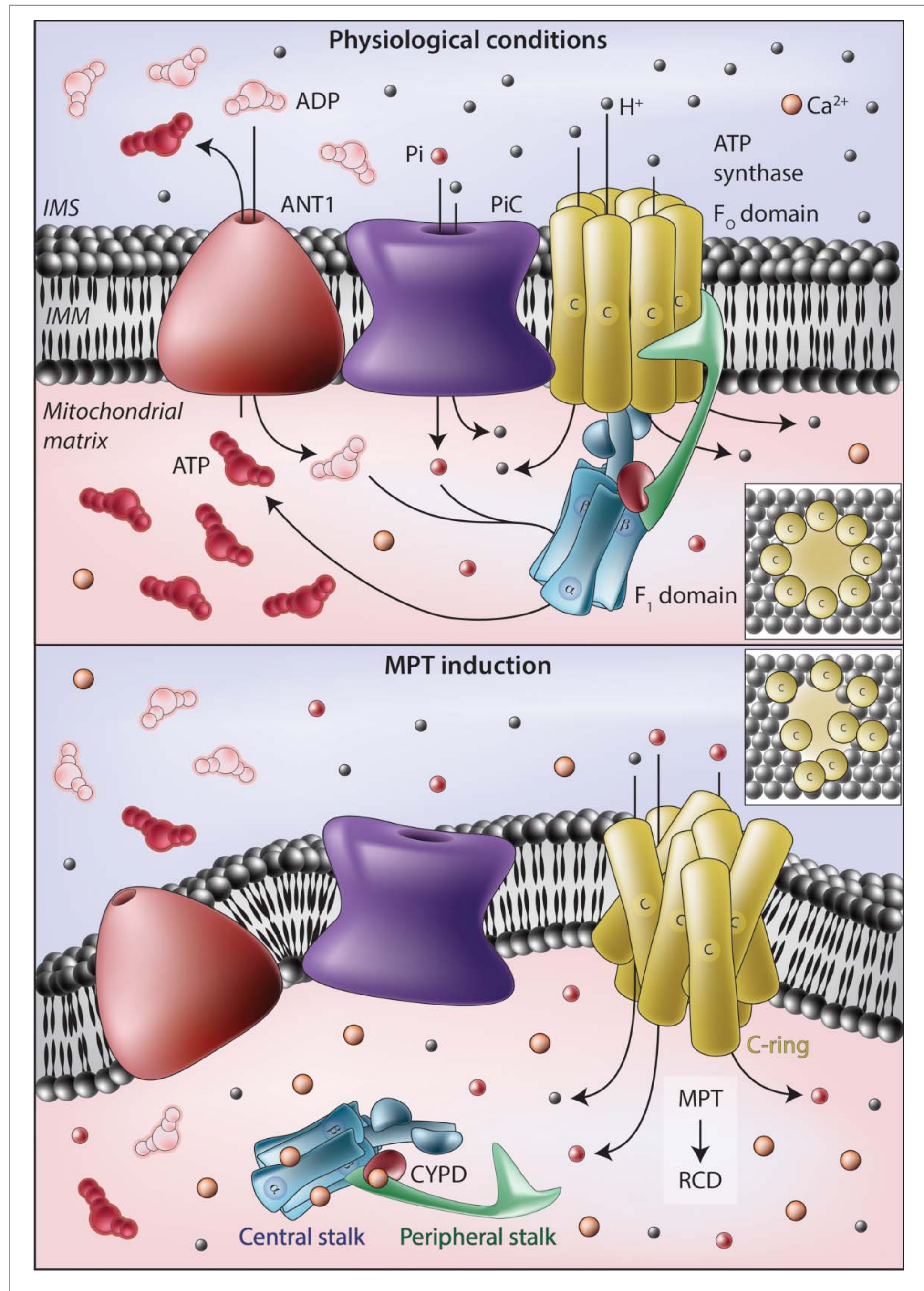

Figure 1. Implication of the ATP synthasome in the mitochondrial permeability transition. (**A**) In physiological conditions, the F_1F_O ATP synthase harnesses the electrochemical gradient established across the inner mitochondrial membrane (IMM) by respiratory chain complexes to produce ATP. The substrates of this reaction, i.e., ADP and inorganic phosphate (Pi), are provided by various members of the solute carrier (SLC) protein family, including SLC25A4, best known as adenine nucleotide translocase 1 (ANT1) and SLC25A3, best known as mitochondrial phosphate carrier (PiC). In particular, ANT1 exports ATP from the mitochondrial matrix in exchange of ADP (both of which are transported along their concentration gradient), whereas PiC operates as an H^+-driven Pi/H^+ symporter. (**B**) In response to oxidative stress or cytosolic Ca^{2+} overload, Ca^{2+} ions accumulate in the mitochondrial matrix and bind to the F_1 domain of the F_1F_O ATP synthase, an activity that may be regulated by peptidylprolyl isomerase F (PPIF, best known as cyclophilin D, CYPD). In these conditions, F_1 domains appear to dissociate from their IMM-embedded interacting partners (F_O domains), allowing c-rings to structurally rearrange and form relatively unselective pores that initiate the mitochondrial permeability transition (MPT). Both ANT1 and PiC have been shown to influence the propensity of cells to undergo MPT-driven regulated cell death (RCD), but the precise molecular mechanisms remain elusive. IMS, mitochondrial intermembrane space.

proteins might be amenable to such manipulation, for at least two reasons. First, it may not be possible to molecularly dissociate the vital and lethal activities of all proteins involved in the initiation or execution of RCD by mutagenesis. Second, the existence of a relatively consistent degree of genetic and epigenetic redundancy may prevent (or at least complicate significantly) the establishment of *bona fide* knock-in models. This latter issue applies to the c subunit of the F_OF_1 ATP synthase, which in the mouse genome is coded by 3 distinct loci (*Atp5g1*, *Atp5g2* and *Atp5g3*).[33] It may therefore be difficult to obtain unequivocal genetic evidence demonstrating the true pathophysiological relevance of c-rings for MPT-driven RCD *in vivo*. Irrespective of these obstacles, it is now clear that the ATP synthasome occupies a central position in the molecular mechanisms that regulate the transition between a cell's life and its death.

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