Biochemical and Biophysical Research Communications 449 (2014) 370–372

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Discussion

The uniporter: From newly identified parts to function

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1. Introduction

Our foray into the molecular biology of the uniporter began with MitoCarta, a high quality inventory of the mammalian mitochondrial proteome we created using protein mass spectrometry, machine learning, and microscopy [1]. To search for genes encoding the uniporter, we were motivated by comparative physiology. It was long known that uniporter current is present in all metazoans tested and even in highly diverged kinetoplastids [2], but, as Ernesto Carafoli and Albert Lehninger had shown decades ago [3], is absent in yeast *Saccharomyces cerevisiae*. Using these taxonomic clues, we devised a comparative genomics strategy to search for human MitoCarta proteins with homologs in kinetoplastids but not in yeast, leading to the identification of MICU1 in 2010 [4].

With MICU1 in hand, we continued to use computational genomics to identify the pore-forming subunit, MCU [5]. We demonstrated that MCU forms oligomers within the mitochondrial inner membrane, we identified a point mutant that retains Ca^{2+} transport but confers resistance to Ru360, the classic inhibitor of the uniporter, and we identified mutations at MCU's evolutionarily conserved acidic residues that abolish Ca^{2+} uptake [5]. De Stefani et al. [6] also leveraged the previous identification of MICU1 and MitoCarta to reach the conclusion that MCU is the pore-forming subunit.

Identification of MCU provided us with a handle for affinity purification of the uniporter holocomplex (uniplex). We used quantitative mass spectrometry to characterize the uniplex in HEK-293T cells and showed it includes MCU and its paralog MCUb, MICU1 and its paralog MICU2, and a distinct protein called EMRE [7]. The exact function of MCUb remains unclear, though RNAi experiments suggest that it is not required for uniporter current [7,8]. EMRE, on the other hand, is a small protein that spans the inner mitochondrial membrane and, like MCU, is essential for uniporter current in human cell lines [7]. MICU1 and MICU2 appear to have regulatory roles [9–12].

The flurry of activity in these first few years of the "molecular era" of uniporter biology has afforded many new insights and observations. Perhaps most importantly, studies now demonstrate that, like most channels, the uniporter corresponds to a macromolecular complex. Here we discuss some of the important questions that are currently debated and will need to be resolved to move the field forward.

2. Composition of the uniplex

The composition of the uniplex is just starting to emerge [7]. The initial discovery of MICU1 opened the doors to the discovery of MCU and their respective paralogs, MICU2 and MCUb. These proteins—MICU1, MCU, and their paralogs—likely represent the core, evolutionarily conserved uniporter machinery, which we predict was a part of the earliest mitochondria [13]. Another protein that we have recently shown to be an essential component of the human uniplex, EMRE, is required for uniporter current and facilitates the interaction of MICU1/2 with MCU [7]. Each of these proteins immunoprecipitates with MCU and has been shown to impact mitochondrial Ca^{2+} uptake. It has also been proposed that other proteins, including MCUR1 and SCaMC-3, are part of the uniplex [14,15]; however, these proteins were not specifically recovered in immunoprecipitation experiments in HEK-293T cells [7]. In fact, MCUR1 immunoprecipitation does not recover MICU1 [14]. It seems possible that these proteins, along with other factors such as LETM1, UCP2/3, and NCLX, modulate mitochondrial Ca^{2+} flux indirectly or independently from the uniplex. Additionally, MICU3 is a paralog of MICU1 and MICU2 that is expressed primarily in the CNS [11]. We have hypothesized that MICU3 participates as a part of the MICU1/2 regulatory complex within the CNS [11].

3. Components necessary and sufficient for conductance

There is now overwhelming evidence supporting the notion that MCU is a pore-forming subunit, but a key question is whether MCU is sufficient for uniporter-mediated Ca^{2+} uptake. In our initial report of MCU we showed that it oligomerizes in the mitochondrial inner membrane [5], point mutations at evolutionarily conserved residues disrupt Ca^{2+} transport, and a single S259A point mutation alters sensitivity of the electrophysiologically defined current to Ru360 [16]. In a separate paper it was suggested that the human

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http://dx.doi.org/10.1016/j.bbrc.2014.04.143
0006-291X/

MCU is sufficient to support mitochondrial Ca^{2+} uptake through planar lipid bilayer experiments [6]. However, the electrophysiological properties reported are distinct from what has been reported in intact mitoplasts and planar lipid bilayers are prone to suffer from artifacts [16,17]. We have recently shown that EMRE is required for MCU-mediated Ca^{2+} uptake in human cell lines [7]. In the absence of EMRE, MCU protein is fully expressed and oligomerizes within the mitochondrial inner membrane, but neither mitochondria nor mitoplasts from human cells lacking EMRE are capable of uptaking Ca^{2+} via a uniporter mechanism. This role of EMRE appears to be distinct from its role in facilitating the interaction of MICU1/2 with MCU since Ca^{2+} uptake is evident in cells lacking MICU1/2 but not in cells lacking EMRE. MCUb also appears not to be required for Ca^{2+} uptake [8]. Therefore, *in vivo*, it appears that human MCU requires EMRE but not MICU1/2 or MCUb for uniporter activity. EMRE, however, appears to be a metazoan-specific protein. Since the uniporter is indeed present in other organisms, it is unclear at present if MCU's requirement for EMRE is specific to metazoans or if there are yet undiscovered functional homologs in other organisms.

4. Localization and topology

The submitochondrial localization and topology of uniplex subunits have been disputed since their initial discovery. While MCU is clearly a mitochondrial inner membrane protein, initial reports were discordant in terms of its topology. In our initial report [5], we examined the protease susceptibility of MCU in mitoplasts, which suggested that MCU is a mitochondrial inner membrane protein with an N-in, C-in configuration, with virtually all of the protein in the mitochondrial matrix, and only a short linker region containing the "DIME" motif facing the IMS. Consistent with this topology, we also showed that the S259A mutant of MCU (which is predicted to face the IMS in this topology) is resistant to the action of Ru360, which is known to be cell impermeant. De Stefani et al. [6], however, reported the opposite topology (N-out, C-out) with virtually all of the protein in the IMS by examining the susceptibility of GFP-tagged MCU to fluorescence quenching. The topology was subsequently clarified using ascorbate peroxidase (APEX) tags on MCU in combination with electron microscopy, clearly supporting an N-in, C-in configuration with most of the protein in the matrix [18].

Although the topology of MCU now appears to be resolved, the submitochondrial localization of MICU1 continues to be debated. Our initial study reporting the discovery of MICU1 did not evaluate submitochondrial localization [4], but a subsequent study showed that MICU1 is likely a peripheral membrane protein, and selective permeabilization followed by limited proteolysis indicated that the endogenous protein responds more similarly to proteins localized to the IMS than the matrix [10]. A recent study, however, reported that GFP-tagged MICU1 does not diffuse away from the inner mitochondrial membrane as quickly as cytochrome c when the outer membrane is permeabilized, suggesting that it is either a matrix-localized protein or more closely associated with the inner membrane than cytochrome c [19]. Each experimental approach has its limitations: proteolysis experiments afford assessment of the endogenous protein but suffer from limitations including possible localization-independent differences in protease susceptibility; diffusion experiments do not require proteolysis but require large tags, overexpression, and their interpretation for peripheral membrane proteins is unclear. Orthogonal methods for evaluating MICU1 localization will be required to gain clarity.

Prior to the discovery of EMRE, a predicted single-pass transmembrane protein, it seemed more intuitive for MICU1 to interact with the complex in the matrix due to the majority of MCU being in the matrix. However, it is now evident that MICU1 could in principle interact with the complex via EMRE and/or the IMS-exposed loops of MCU/MCUb. In fact, EMRE is required for the interaction of MICU1/2 with MCU by co-immunoprecipitation [7]. Thus, MICU1 may interact with the complex on either side of the inner mitochondrial membrane. Our experiments employing limited proteolysis indicate that the localization and topology of MICU2 and MCUb align with their paralogs [7]. While it is clear that EMRE is an integral inner membrane protein with a single predicted transmembrane domain [7], its orientation has not yet been investigated.

Thus, while the topology of MCU is now accepted to be N-in, C-in, orthogonal methods will be required to fully establish the submitochondrial localization of MICU1 and MICU2 and determine the orientation of EMRE. These experiments will help us formulate a model for the macromolecular organization of the uniplex.

5. Regulation by MICU1/MICU2

Our understanding of uniporter regulation by MICU1 and MICU2 has been fast evolving since their discovery. Initially, measurements of matrix Ca^{2+} revealed that MICU1 knockdown cells show blunted increase in matrix Ca^{2+} signal upon histamine stimulation [4], which we interpreted as a reduction of Ca^{2+} uptake into mitochondria. This observation remains consistent with subsequent reports of MICU1 knockdown in cell lines [9–11,20]. Silencing of MICU1 and MICU2 *in vivo* in mouse liver, too, leads to a Ca^{2+} uptake deficit based on clearance assays [11]. However, it is important to note that in mouse liver, MICU1 and MICU2 silencing resulted in decreased MCU protein levels as well, obfuscating the true role of these proteins in the Ca^{2+} uptake deficit. While the aforementioned observations could be used as evidence for an activating role of MICU1 and/or MICU2, since then it has been clearly shown in two independent studies that loss of MICU1 in at least some cell lines leads to increased Ca^{2+} uptake when mitochondria are exposed to low [Ca^{2+}], suggesting a gatekeeping role for MICU1 [9,10]. While both of these subsequent studies agree that MICU1 sets the threshold for uniporter-mediated Ca^{2+} uptake, some basic mechanistic details proposed have been disputed—most fundamentally, whether Ca^{2+} uptake is inhibited by apo- or Ca^{2+}-bound MICU1. Specifically, discordant effects of locking MICU1 in the apo-state through mutations in the EF hands has led to contradictory models for MICU1 gatekeeping function. One model suggests that MICU1 inhibits Ca^{2+} uptake in the Ca^{2+}-bound state [9], while another model involves MICU1 inhibition in the apo-state [10]. Additionally, the potential role of MICU1 as an activator in MCU-mediated Ca^{2+} uptake has also been proposed in addition to its inhibitory function [10] or as its sole function [20].

Many factors may contribute to the discrepancies between studies of MICU1 function, which have yet to be fully resolved. First, matrix Ca^{2+} measurements have been misleading in the context of MICU1 manipulation, which may be due to differences in baseline matrix Ca^{2+} content or differences in Ca^{2+} buffering capacity in response to loss of MICU1 [4,9,10]. Second, particularly in studies of permeabilized cells, buffer composition may have other important implications. For example, the presence or absence of Mg^{2+} has been shown to result in the presence or absence of MICU1-dependent cooperativity of uniporter-mediated Ca^{2+} uptake [10]. Third, different cell or tissue types explored can contribute to the discrepancies: for example, stability of the uniplex proteins in response to loss of one component has been shown to be cell- and tissue-type dependent [11]. Fourth, many of the earlier studies of MICU1 did not consider MICU2, and as we have shown, the two form a complex, and overexpression of exogenous MICU1 or MICU2 proteins in knock down cells leads to stabilization of

residual wild type protein, whose levels are important factors determinant of the observed phenotype [11]. Thus, interpretation of data from RNAi-based knock down experiments can be complicated.

To overcome these problems, we recently used genetically engineered MICU1 or MICU2 knock out cell lines and assessed their function in HEK-293T cells [12]. These experiments enabled us to assess the function of each protein individually, allowing us to conclude that MICU1 and MICU2 operate together to regulate the uniporter. In fact, functional and biochemical experiments suggest a model in which MICU1 and MICU2 operate "in series": MICU2 and MCU co-immunoprecipitate each other only with MICU1 present, whereas interaction of MICU1 with MCU is not dependent on MICU2; moreover, in order for the EF hand mutant of MICU2 to inhibit Ca^{2+} uptake, presence of MICU1 is required, but not vice versa. This finding makes it particularly important to take MICU2 into account while studying MICU1 function, since they appear to functionally complement each other. In this study, we showed that either MICU1 or MICU2 locked in the apo state (with EF hand mutations) is capable of inhibiting Ca^{2+} uptake [12]. Thus, in support of the MICU1 model proposed by Csordás et al. [10], we find that both MICU1 and MICU2 inhibit Ca^{2+} uptake in the apo state and that MICU1 and MICU2 operate together to gate the uniporter [12]. In fact, it has been recently proposed that MICU1 and MICU2 may even share a disulfide bond [20]. However, the role of MICU1 and MICU2 might be more complicated. For example, matrix Ca^{2+} measurements have suggested that loss of MICU1 may have a secondary impact on buffering [10]. Furthermore, loss of MICU1 appears to lead to decreased Ca^{2+} uptake in a cell and tissue-dependent manner, which in some cases may be due to reduction of MCU. Hence, clarifying the mechanisms and precise roles of MICU1 and MICU2 will continue to require further investigation.

6. The path forward

In the first few years since the discovery of the founding uniplex member, MICU1, and the pore forming subunit, MCU, this field has seen an explosion of advancements. Much remains controversial, unclear, or unexplored. Mapping the interaction domains of each uniplex component will be important work in the coming years in order to draw a more detailed macromolecular map. This information could be greatly facilitated by elucidating 3-dimensional structures in addition to learning the stoichiometry of each component of the complex. Importantly, MICU1's 3D structure has recently been investigated by X-ray crystallography. However, it will be important to take a step further by crystallizing MICU1 with and without Ca^{2+} bound using the same sequence and in addition by determining how MICU2 resides within this complex. Additionally, the crystal structure suggests that MICU1 may form a dimer or even a hexamer, which will be important to clarify as we attempt to link structure to function. Determining the physiological contexts under which uniporter-mediated Ca^{2+} uptake operates will also be important in the coming years, which may additionally help us understand why we see tissue- and cell-type-specific differences when studying the uniporter. Finally, whole organism studies have already started to reveal surprising aspects of the physiology of its components [21–23] and promise to provide valuable insights into the role of this channel complex in disease.

Acknowledgments

V.K.M. is an Investigator of the Howard Hughes Medical Institute. K.J.K. was supported by a graduate research fellowship from the National Science Foundation. Y.S. was supported by the Helen Hay Whitney Foundation.

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