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Genetic Manipulation of The Cardiac Mitochondrial Phosphate Carrier does not affect Permeability Transition

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Abstract

The Mitochondrial Permeability Transition (MPT) pore is a voltage-sensitive unselective channel known to instigate necrotic cell death during cardiac disease. Recent models suggest that the isomerase cyclophilin D (CypD) regulates the MPT pore by binding to either the F₀F₁-ATP synthase lateral stalk or the mitochondrial phosphate carrier (PiC). Here we confirm that CypD, through its N-terminus, can directly bind PiC. We then generated cardiac-specific mouse strains overexpressing or with decreased levels of mitochondrial PiC to assess the functionality of such interaction. While PiC overexpression had no observable pathologic phenotype, PiC knockdown resulted in cardiac hypertrophy along with decreased ATP levels. Mitochondria isolated from hearts of these mouse lines and their respective non-transgenic controls had no divergent phenotype in terms of oxygen consumption and Ca²⁺-induced MPT, as assessed by swelling and Ca²⁺-retention measurements. These results provide genetic evidence indicating that the mitochondrial PiC is not a critical component of the MPT pore.

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Keywords

Mitochondrial permeability transition; mouse genetics; cyclophilin-D; mitochondrial phosphate carrier

1. Introduction

In cardiomyocytes, mitochondria are responsible for key metabolic pathways that will determine the fate of the cell. The normal activity of these organelles is essential for processes such as fatty acid oxidation, porphyrin synthesis, ion homeostasis, and oxidative phosphorylation. However, mitochondrial dysfunction is seldom compatible with cellular viability and is often a cause of myocardial pathologies. Conditions threatening cardiac mitochondrial homeostasis such as ischemia/reperfusion injury [1], diabetes [2], or the damage induced by chemotherapeutics [3] are often associated with increases in unselective mitochondrial permeability to water, ions and metabolites. These changes in permeability induce mitochondrial respiratory chain collapse, oxidative stress, and ultimately swelling and rupture of the organelle, thereby triggering death of the cardiomyocyte [4].

Opening of the MPT pore, a non-selective channel thought to span the inner mitochondrial membrane, is known to be the underlying cause of the permeability changes that initiate mitochondrial-induced cell death [5]. For over 2 decades, the MPT pore was thought to be the consequence of a redox-induced conformational change of the adenine nucleotide translocase (ANT) in the inner membrane catalyzed by the chaperone cyclophilin-D (CypD) in the matrix [6]. However, studies in gene-knockout mice have cast doubt on the validity of this model as mice lacking ANT or CypD still exhibit an MPT response, albeit with diminished sensitivity to ANT and CypD ligands respectively [7–10]. Ca^{2+} -induced MPT pore is effectively modulated by accessory CypD overexpression or in gene-targeted mice [9]. Thus ANT and CypD appear to be regulators of the MPT pore rather than the pore itself. More recent hypotheses propose that the F_1F_0 -ATP synthase or the mitochondrial phosphate carrier (PiC) may instead form the MPT pore, where binding of these proteins to CypD is affected by CsA [11–15]. However, both of these models still need extensive testing and genetic validation as CypD may bind to other proteins such as Hsp90/TRAP1, Bcl-2 and C1qbp [16–18].

Here, we investigated the role of PiC in comprising the MPT pore through structural, genetic and bioenergetic approaches to further understand the role of this unselective mitochondrial pore in cardiac cell death and the progression of cardiovascular disease. We confirm that PiC binds to CypD and map the interaction site to the N-terminus of CypD N-terminus. To assess the functionality of such interaction, we generated cardiac-specific transgenic mouse strains overexpressing PiC from 4 to 6 fold and a PiC knockdown strain where the carrier expression was decreased ~ 60%. PiC overexpression, either alone or in conjunction with CypD upregulation, resulted in no obvious phenotypic pathology *per se*. PiC knockdown resulted in cardiac hypertrophy with decreased fractional shortening thus partially recapitulating the outcome of PiC deficiency observed in humans [19,20]. Isolated cardiac mitochondria from the lines either overexpressing or underexpressing PiC levels still

displayed a typical Ca^{2+} -induced MPT pore response with comparable characteristics. Our results suggest that the level of expression of PiC in mouse mitochondria has little effect on classical readouts of the MPT pore opening. This argues against a main role of PiC as the MPT pore-forming component.

2. Experimental Procedures

2.1 Animals

All the experiments involving mice were approved by the University of Missouri Animal Care and Use Committee and conformed to NIH guidelines for the use and care of animals. To generate transgenic (TG) mice, mouse PiC cDNA was inserted in the α -myosin heavy chain (MHC) promoter cassette and injected into fertilized FVB/N oocytes. PiC shRNA TG mice were generated as described previously [21]. Briefly, an shRNA against mouse PiC (Open Biosystems) was subcloned downstream of a tetracycline responsive minimal CMV promoter in the pTMP vector (Open Biosystems). The transgene was linearized with *Bgl*II and *Age*I and injected into fertilized FVB/N oocytes. The resultant mice were then crossed with cardiac-specific tTA driver mice [22] to express the shRNA, the target sequence of which was 5'-tcaacaagcagattcagtc-3'. All experiments were performed when the mice were 2 months of age and comparisons were made against respective non-transgenic (NTG) littermates.

2.2 Echocardiography

Echocardiograms were performed using a GE Vivid 1 ultrasound system with a 12-mHz transducer. Analysis was performed offline using GE EchoPac Software.

2.3 Mitochondrial Isolation and Assays

Heart mitochondria were prepared from the different mouse lines by differential centrifugation in sucrose-based medium, as previously described [9,23]. Mitochondria were resuspended in swelling buffer (120 mM KCl, 10 mM Tris at pH 7.4 and 5 mM KH_2PO_4) at 0.20 mg/mL. MPT was measured spectrophotometrically as a decrease in light scattering at 520nm and was induced by the addition of 250 μM CaCl_2 to de-energized mitochondria. In calcium retention capacity (CRC) experiments, extramitochondrial Ca^{2+} was measured fluorimetrically using 1 μM Calcium Green-5N. Mitochondria were resuspended in swelling buffer supplemented with 10mM succinate to a concentration of 0.125 mg/mL in a final volume of 1mL. 1 μL from a stock solution of 2.5mM CaCl_2 was added each minute until an increase in fluorescence due to Ca^{2+} -release was detected. Oxygen consumption was measured polarographically at 25°C using a Clark-type electrode in the medium used for swelling measurements supplemented with 1mM MgCl_2 and either 5mM glutamate/5 mM malate or 10mM succinate. State 3 was initiated by adding 200 μM ADP to the reaction mixture. ATP levels from mitochondrial fractions were determined using the CellTiter-GLO luminescence assay from Promega according to the manufacturer's instructions.

2.4 Adult Mouse Cardiomyocyte Isolation and Evaluation of CRC

Mice were anesthetized with pentobarbital sodium (60mg/kg) and after deep anesthesia was confirmed the chest cavity was opened and hearts were rapidly (~ 30s) removed and placed

in 4 °C Ca^{2+} -free physiological saline solution (PSS, 135mM NaCl, 5mM KCl, 2mM CaCl_2 , 1mM MgCl_2 , 10mM D-glucose, 10 mM Hepes, pH 7.4). Excised hearts were immediately cannulated via the aorta and retrogradely perfused with Ca^{2+} -free PSS containing 2U/ml heparin for 10 minutes, followed by 15–16 minute perfusion with a minimal essential medium (MEM)-based enzymatic isolation solution supplemented with: 10mM NaHCO_3 , 2mM Na-Pyruvate, 10 mM NaHEPES, 10mM HEPES, 8mM Taurine, 20 μM CaCl_2 , 50,000 U/L Penicillin-Streptomycin, and 22.5 $\mu\text{g}/\text{mL}$ Liberase Blendzyme TH (Roche Applied Science), pH 7.35. Hearts were removed from the perfusion unit and placed in a MEM-based solution supplemented with 10mM NaHCO_3 , 2mM Na-Pyruvate, 10mM NaHEPES, 10mM HEPES, 40 μM CaCl_2 , 50,000 U/L PenStrep, and 10mg/mL bovine serum albumin. The left-ventricle and septum were minced, agitated, and filtered (200 μm nylon mesh) to obtain isolated cardiomyocytes. Cardiomyocytes were counted (30,000 per experiment) and resuspended with 1 mL of buffer containing 120 mM KCl, 10 mM Tris at pH 7.4, 1 mM KH_2PO_4 , 10 mM succinate, 20 μM EDTA and 0.5 μg digitonin. CRC was measured as described for isolated mitochondria by detecting changes in the fluorescence of 1 μM Calcium Green-5N at 530nm.

2.5 Western blotting

Cardiac whole tissue and mitochondria were homogenized in buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1mM EDTA, and 1% Triton-X100. Proteins were resolved by SDS-PAGE using 10% acrylamide, transferred onto PVDF membranes, and immunoblotted using the following commercially available antibodies: anti-ATP synthase, anti-CypD, anti-GST, and anti-VDAC from Abcam; and anti-ANT from Santa Cruz. The polyclonal PiC antibody was custom made for us by Yenzyme. Membranes were then incubated with the appropriate alkaline phosphatase-linked secondary antibody (Cell Signaling) and visualized by enhanced chemifluorescence (Amersham).

2.6 Pulldowns

Mature CypD and the various CypD truncation mutants were amplified by PCR and subcloned into the pGEX-4T1 vector. The resultant recombinant proteins were then incubated with cardiac mitochondrial lysates plus glutathione sepharose beads overnight at 4°C. After washing 3 times with homogenization buffer the complexes were then subjected to Western blotting for PiC and GST. For the direct interaction studies, His-tagged recombinant CypD (ProSpec) was incubated with GST-PiC (Abnova) for 15 min at RT in PBS plus 0.1% NP-40, and the resultant complexes purified using Co^{2+} affinity columns or glutathione sepharose.

2.7 Protein modeling and docking

The murine mitochondrial PiC model was generated on I-TASSER [24] using the mitochondrial ADP/ATP carrier PDB model 1OKC as template. The available CypD PDB model 2BIT was used for further docking simulations with the PiC model using RosettaDock [25]. The starting conformation for *local* docking search was set by placing CypD near the PiC domains corresponding to the mitochondrial matrix portion of the

carrier. All output models were considered for further analysis and were rendered using PyMol [26].

2.8 Statistical analyses

Data are presented as mean $\pm$ s.e.m. Statistical evaluation between 2 groups was performed by unpaired *t*-tests. For 3 groups, one-way ANOVA with Scheffe's *post hoc* test was used. A *P* value <0.05 was considered statistically significant.

3. Results

3.1 Mapping the interaction between PiC and CypD

It was previously shown that PiC interacts with the MPT pore regulatory protein CypD in a CsA-dependent manner [14]. Consequently, we aimed to reproduce such findings and to further decipher the nature of this potential interaction. Following the incubation of mouse cardiac mitochondrial lysates with recombinant GST (control) or GST-CypD, the resulting complexes were subjected to western blotting against PiC. The results show an increased detection of PiC in the samples mixed with recombinant GST-CypD as compared to GST alone (Fig. 1A). To assess whether such an interaction is direct we incubated recombinant GST-PiC with increasing amounts of His-tagged recombinant CypD (Fig. 1B). The resulting complexes were bound to GST-tagged beads and “pulled down” before extensive washing. The ability of the GST-PiC to pull down CypD indicated that the 2 proteins could directly bind to one another. This was confirmed using the antithetical experiment where recombinant His-tagged CypD was incubated with increasing amounts of GST-PiC and the resulting complexes were pulled down using a Co^{2+} resin (Fig. 1B). These results demonstrate that CypD and PiC interact directly in a specific and concentration-dependent manner. To further gain insight into the binding domains involved in the PiC-CypD interaction, we generated GST-CypD fusion proteins with sequential deletions on the C-terminal portion of the protein (Fig. 1C) and repeated the pulldown experiments under the same conditions as those shown in Fig. 1A. PiC was able to bind all of the CypD fusion proteins with the exception of the chimera consisting of only first 40 amino acids of the N-terminal portion of the mature CypD protein (Fig. 1C). This implies that PiC binds to the region between residues 70 and 110 in CypD.

We next sought to validate such findings by performing *in silico* docking simulations between an *ad hoc* PiC model we generated and a deposited CypD Protein Data Bank (PDB) model (2BIT) using Rosetta Docking Software [25]. The results suggest that CypD binds to a discrete patch of residues around arginine 304 located on the matrix portion of murine PiC in all output models (Fig. 1D). According to these results however, CypD can dock to PiC either from the “backface” [27] of the protein on 60% of the structures or the CsA-binding domain on 40% of the structures (Fig. 1D). To further explain the nature of such interactions, CypD qualitative electrostatic potential rendering of these models showed a predominant acidic surface (red) on the backface of the isomerase while the “gatekeeper” [27] and the CsA-binding domain present a mixed distribution of charges (red, blue and white) (Fig. 1E). Virtual deletion of residues 70 to 110 considerably impacts the backface, the gatekeeper and the CsA-binding domains of CypD potentially explaining why PiC

binding is decreased when CypD lacks these residues. Conversely, PiC electrostatic potential rendering shows a basic patch of residues (blue) around the putative binding site located in the “matrix side” of the carrier (Fig. 1E).

3.2 Functional characterization of cardiac-specific PiC transgenic mouse lines

Given the specificity of the interaction between CypD and PiC reported here and proposed by others [13,14,28], we reasoned that PiC may be a key component of the MPT pore. Consequently, we sought to determine whether cardiac-specific PiC manipulation, driven by the α myosin heavy chain (α -MHC) promoter was sufficient to cause MPT and cardiac pathology, as we have previously shown with cardiac CypD overexpression [9]. We successfully generated 2 transgenic lines that exhibit ~4.5 and 6 fold increased expression levels of PiC in the heart (lines TG1 and TG2, respectively). Other putative MPT complex proteins such as ANT, CypD, and ATP synthase were unchanged (Fig. 2A,B). Interestingly VDAC expression was increased 1.50 ± 0.03 and 1.58 ± 0.16 -fold in the TG1 and TG2 lines, respectively, when compared to NTG controls (Fig. 2B). Gravimetric analysis of excised hearts from TG1 and TG2 lines showed no obvious differences in terms of size and heart weight/body weight ratio (Fig. 2C,D). Echocardiographic measurements of mice from TG1 and TG2 lines detected no apparent signs of pathology with no changes observed in fractional shortening and chamber size between the NTG and TG mice (Fig. 2E,F,G).

We isolated mitochondria from hearts of NTG, TG1 and TG2 mice and performed oxygen consumption experiments to determine respiratory chain function (Fig. 3A) [29]. As an indicator of mitochondrial coupling, we measured the ratio between phosphorylating and basal respiration rates (RCR) in the presence of complex I or complex II substrates (Fig. 3A). Mitochondria from NTG mice presented a RCR of ~10 in the presence of complex I substrates and ~3 with succinate as electron donor. Mitochondria isolated from TG1 and TG2 lines presented similar values suggesting that PiC upregulation does not impact the coupling efficiency of isolated mitochondria (Fig. 3A). We next measured steady state mitochondrial ATP levels and measured no statistical differences between the three lines (Fig. 3B).

3.3 PiC upregulation does not affect Ca^{2+} -induced MPT in isolated cardiac mitochondria

In order to assess the role of PiC upregulation on MPT pore activity, we performed swelling experiments on isolated cardiac mitochondria under de-energized conditions to avoid the interference of membrane potential, ATP/ADP ratio and rates of calcium cycling that can modulate the MPT pore indirectly [28]. Under these conditions, isolated mitochondria from hearts of TG1 and TG2 mice swelled to the same extent as NTG mitochondria, indicating an intact MPT response (Fig. 3C). We next measured the Ca^{2+} -retention capacity of cardiac mitochondria from TG1 and TG2 mice (Fig. 3D). This measure is a robust indicator of MPT pore function as it provides a quantitative way of determining the Ca^{2+} concentration required to achieve pore opening. Under normal conditions, NTG mitochondria presented an uptake of 147 ± 8 nmol Ca^{2+} /mg protein (Fig. 3D,E). This value was increased to 368 ± 8 nmol Ca^{2+} /mg of mitochondrial protein when in the presence of $1 \mu\text{M}$ cyclosporine-A (CsA), a known desensitizer of the MPT pore. In agreement with the swelling results, Ca^{2+} retention in the PiC overexpressing lines was statistically similar to the NTG line with a

value of 115 ± 5 nmol Ca^{2+} /mg protein and $P < 0.053$ in mitochondria from the TG2 line (Fig. 3D,E). Overall, these results indicate that PiC overexpression does not overtly affect MPT pore dynamics.

To further rule out the possibility that overexpression of PiC is not sufficient to drive MPT pore opening due to stoichiometry differences with cardiac CypD (assuming the MPT pore opens when the PiC-CypD complex occurs whereas PiC alone retains its physiological role) we generated a double transgenic strain (DTG) by crossing the TG1 line with the previously characterized cardiac-specific “low” overexpressing CypD TG line (Fig. 4A) [9]. Again, we saw a similar increase in VDAC expression in the DTGs (1.77 ± 0.03 -fold) that we saw in the single TG1 hearts, but no change in ANT or ATP Synthase levels (Fig. 4A). Phenotypically, we observed no changes in the heart weight/body weight ratio in between the NTG, TG1, CypD TG and DTG lines (Fig. 4B,C). Furthermore, we isolated mitochondria from the DTG line and detected a decreased swelling pattern after $250 \mu\text{M}$ Ca^{2+} addition compared to NTG mitochondria (Fig. 4D). However, given the previously reported alterations in mitochondrial architecture in the CypD TG line [9], we decided to measure the Ca^{2+} -retention capacity of NTG and DTG mitochondria to further confirm or reject a possible MPT pore effect based on the swelling data shown above. Under control conditions (absence of CsA), the Ca^{2+} -retention of NTG and DTG lines was statistically similar (Fig. 4E). We did measure a decreased Ca^{2+} -retention in the presence of $1 \mu\text{M}$ CsA in the DTG line, however we attribute this to an increase in free CypD in this line as treatment with a greater concentration of CsA ($5 \mu\text{M}$) was able to increase the Ca^{2+} -retention capacity further in these mitochondria (Fig. 4E). Together, the results suggest cardiac PiC upregulation does not affect the MPT pore.

3.4 Generation of a mouse strain with cardiac-specific PiC silencing

To further assess the potential contribution of PiC on the MPT pore and cardiac pathology, we generated a mouse line with inducible cardiac-specific shRNA-mediated repression of PiC. To achieve this, we crossed a “driver” transgenic line in which the tet-repressor protein (tTA) is constitutively expressed in the heart using the traditional α -MHC promoter with a “responder” transgenic line (shTG) containing a PiC-specific shRNA downstream of a minimal CMV promoter with a tet responsive element. Single transgenic tTA and shTG mice exhibited the same levels of PiC expression as NTG mice. However, crossing the tTA and shTG mice lead to a ~60% reduction in cardiac PiC levels (Fig. 5A,B). There were no alterations in the expression of ANT, CypD, ATP synthase or VDAC in any of the mouse lines (Fig. 5A). Interestingly, gravimetric assessment of hearts from NTG and tTA+shTG mice showed an increased HW/BW ratio in the PiC-depleted hearts (Fig. 5C,D), suggesting a hypertrophic response reminiscent of PiC deficiency in humans [20]. In addition, hemodynamic studies on hearts from tTA+shTG mice showed a small but significant reduction in fractional shortening (Fig. 5E). Chamber size however was unaffected in the tTA+shTG mice (Fig. 5F,G).

3.5 PiC knockdown does not affect Ca^{2+} -induced MPT in isolated cardiac mitochondria or isolated cardiomyocytes

Given the importance of Pi availability to drive ATP synthesis by F_1F_0 -ATP synthase, we first sought to determine the impact of PiC gene silencing on mitochondrial respiratory chain

activity of the tTA+shTG line (Fig. 6A). In the presence of complex I substrates, isolated cardiac mitochondria from the tTA+shTG line presented a non-significant decrease in the respiratory control ratio compared to its NTG counterpart. Complex II-dependent respiration yielded identical results between the NTG and the tTA+shTG lines (Fig. 6A). However, further assessment of steady-state ATP levels in the tTA+shTG line showed a significant decrease compared to the NTG strain suggesting impaired energetics at baseline (see discussion, Fig. 6B). We next assessed MPT pore activity in isolated cardiac mitochondria from the NTG and tTA+shTG under de-energized conditions and found a Ca^{2+} -induced swelling pattern that was comparable between mitochondria from both genotypes (Fig. 6C). To further test the Ca^{2+} -threshold to achieve MPT pore opening, we performed Ca^{2+} -retention capacity experiments in NTG and tTA+shTG mitochondria (Fig. 6D). In agreement with previous results in a cell culture model [28], the required Ca^{2+} addition to achieve Ca^{2+} -release was nearly identical in both lines. In the presence of CsA, Ca^{2+} -retention was still increased in both groups, just slightly less in cardiac mitochondria from tTA+shTG mice (Fig. 6E). As our mitochondrial preparation contained mitochondria from non-cardiomyocyte cells, we wanted to verify these results directly in isolated ventricular cardiomyocytes. Myocytes were prepared from NTG and tTA+shTG mouse hearts, permeabilized with digitonin, and subjected to the Ca^{2+} -retention capacity assay. As shown in Fig. 6F,G there was no difference in Ca^{2+} -retention between the normal and the PiC knockdown myocytes.

4. Discussion

Heart disease is the leading cause of premature death among humans worldwide. Unfortunately, the knowledge available of the mechanisms underlying such dysfunctional states is relatively modest. The discovery that mitochondrial dysfunction is often associated with cardiac disease provides an interesting opportunity to unveil the molecular mechanisms acting behind these pathologies. In particular, the discovery that cardiac dysfunction is often the consequence of MPT pore opening provides a therapeutic window to understand and design feasible treatment approaches to selectively target this phenomenon [30]. In this work we show that mitochondrial PiC is a *bona fide* CypD binding protein. Interaction between these proteins was detected using either recombinant CypD or PiC in a concentration-dependent manner. In addition, pulldown experiments with GST-CypD fusion proteins with sequential deletions on the C-terminal portion of the protein allowed us to determine that CypD residues 70–110 are required to bind PiC. This is of special relevance with regards to two potential regulatory residues of CypD-dependent MPT, i.e. C203 and R96 [9,31]. The domain we propose to be required for CypD binding to PiC would only compromise R96 located in the CsA-binding domain. This is in apparent contrast with our docking simulations where binding can be achieved either from the backface of CypD or the CsA-binding domain. However, these two potential binding conformations could explain why binding between CypD and PiC is not completely suppressed with CsA [13] as the backface of the protein would still be free to bind PiC even in the presence of CsA.

To assess the role of PiC in the MPT we successfully generated transgenic mouse strains either overexpressing or downregulating PiC in a cardiac-specific fashion. Although PiC overexpression resulted in no apparent changes in phenotype, ~60% knockdown of PiC

resulted in a significant increase in heart size, with a small but significant decrease in fractional shortening. Thus, PiC downregulation resulted in a hypertrophic phenotype mildly recapitulating the outcome of PiC deficiency reported in humans [19,20]. Although PiC upregulation or knockdown resulted in no change in mitochondrial respiratory coupling, there was a decrease in steady-state ATP levels in the tTA+shTG line. This suggests that tTA+shTG mitochondria are still able to remain coupled under appropriate experimental conditions, whereas their energetic state at baseline is disturbed and could explain the hypertrophic phenotype. That being said, it is difficult to envision how cardiac mitochondria cope with the downregulation of such an important protein required for oxidative phosphorylation. Based on our results, we conclude that 60% of PiC repression is still sufficient to support a compensated phenotype. This diseased but compensated phenotype due to PiC silencing may result from a combination of Pi import from other mitochondrial carriers such as the dicarboxylate carrier [32,33], as well as from neuroendocrine resetting of the heart as a means of attempting to maintain cardiac output or functional reserve, which is known to be associated with cardiac hypertrophy [34].

PiC overexpression resulted in an MPT response with virtually indistinguishable characteristics to that seen in mice with normal PiC levels. These data suggest that elevated PiC expression levels are not sufficient to increase MPT pore activity in the myocardium. However, it is possible that overexpressing PiC simply disrupts the PiC:CypD stoichiometry. To rule this out we crossed the PiC TGs with CypD TGs to see if a concomitant increase was required to alter MPT responsiveness. However, these animals still exhibited a normal MPT response at baseline, albeit with a decreased response to CsA. To our surprise, we also failed to observe any change in MPT in PiC-depleted cardiac mitochondria or cardiomyocytes. These data are consistent with a previous study demonstrating that MPT in HeLa cells was unaffected by an ~70% knockdown of PiC [28]. The simplest explanation is that PiC is not necessary for MPT pore formation, as also suggested for mitochondrial unselective permeabilities in model organisms [35]. Certainly, we would expect that even a modest reduction in PiC would have a significant effect on MPT by being the pore-forming unit or at least a key regulatory component such as CypD. For example, a 50% loss of CypD was sufficient to attenuate Ca^{2+} swelling in heart, liver and brain mitochondria [9]. It is conceivable that the remaining PiC protein can still result in a normal MPT response or that another protein can take its place. However, given that a constitutive knockout is likely lethal [20], tissue-specific and conditional deletion of the *Slc25a3* gene may be required to reduce PiC levels beyond what we have achieved.

In this regard, while this manuscript was revision, Kwong et al [40] described the effects of cardiac-specific deletion of *Slc35a3*. They found that almost complete (~90%) abrogation of PiC expression had a moderate inhibitory effect on Ca^{2+} -induced MPT, but with no effect against atractyloside-induced MPT. Moreover this inhibitory effect was lost when P_i was replaced in the assay buffer with arsenate. Together these results are consistent with ours in indicating that the PiC does not play a major role in MPT pore formation. That being said, depletion of PiC did offer a modest but significant protection against Ca^{2+} - and ischemia/reperfusion-induced cell death [40], suggesting that PiC may still be a regulator of the MPT pore. The differences between our study and theirs are several-fold. First, we used a

different approach to deplete the heart of PiC. Whereas the study by Kwong et al utilized a Cre-Lox approach to delete the first 2 coding exons of the *Slc25a3* gene [40], we transgenically overexpressed a cardiac restricted, PiC-specific shRNA. In addition to the loss-of-function studies, we also examined the effects of PiC overexpression on MPT and cardiac function and found no effect on MPT responsiveness. Finally, we also examined the physical interaction between CypD and PiC, demonstrating that the two proteins could indeed directly bind one another.

Since its initial discovery, the MPT pore's molecular componentry has been an intense matter of hypotheses and debate [36]. Putative pore-forming components with MPT pore-like electrophysiologic behavior such as VDAC and ANT [37,38] have been discarded on the basis of genetic tests [7,23]. Other proposed candidates such as dimeric ATP synthase [11,12] or the mitochondrial protein import machinery [39] are still waiting to be validated by genetic means. Although we provide evidence showing that genetic manipulation of PiC does not affect the MPT as opposed to the effects seen upon differential expression of regulatory CypD, we do not rule out the possibility that this carrier may be interacting within the periphery of the pore as it selectively binds CypD.

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Abbreviations

ANT	adenine nucleotide translocase
CsA	cyclosporine-A
CypD	cyclophilin D
DTG	double transgenic
GST	glutathione S-transferase
MHC	α -myosin heavy chain
MPT	mitochondrial permeability transition
NTG	non-transgenic
PDB	protein data bank
PiC	mitochondrial phosphate carrier
RCR	respiratory control ratio
shTG	cardiac-specific shRNA transgenic
TG	transgenic
tTA	tet-repressor protein

VDAC voltage dependent anion channel

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Highlights

- The phosphate carrier (PiC) has been implicated as a key component of the MPT pore.
- We found that the PiC could directly bind to the MPT pore component cyclophilin-D.
- Yet mice with cardiac-specific expression of PiC exhibited a normal MPT response.
- Mice with cardiac-specific knockdown of PiC also exhibited a normal MPT response.
- Consequently, the PiC does not appear to be a critical component of the MPT pore.

Figure 1.

PiC directly interacts with CypD. (A) Recombinant GST and GST-CypD were incubated with mouse heart mitochondrial lysates as described in “Methods” and the resulting complexes were “pulled down” using GSH-beads and immunoblotted for PiC. (B) Recombinant GST-PiC was incubated with increasing amounts of His-tagged CypD (His-CypD) and the resulting complexes were immunoblotted for CypD. In addition, His-CypD was incubated with increasing amounts of GST-PiC and the resulting complexes were purified using Co^{2+} -linked beads. (C) Schema illustrating GST-fused mature CypD peptide fragments with sequential deletions on the C-terminus. These fusion proteins were incubated with mouse heart mitochondrial lysates and the resulting complexes were pulled down and detected by western blotting. (D) PiC and CypD models were docked as detailed in “Methods” and the output complexes were grouped into 2 different families where CypD “backface” binds a discrete domain on helix 6 of PiC and CypD CsA-Binding Domain docks the same discrete domain on helix 6 of PiC. CypD residue C203 is shown in sphere projection. (E) CypD and PiC models shown colored by qualitative electrostatic potential. The scales of the potentials are roughly the same for all models (average potential: ± 54 kBT/e). All surfaces were calculated using the protein contact potential function in PyMol.

Figure 2.

Functional characterization of cardiac-specific PiC transgenic mouse lines. (A) Western blot showing enhanced expression of PiC in cardiac mitochondria from TG1 and TG2 mice but not ANT, CypD, ATP synthase (AtpS) and VDAC. (B) Quantification of PiC protein levels as assessed by standard densitometry. (C) Representative images of hearts excised from the indicated NTG and transgenic lines. (D) Gravimetric assessment of NTG, TG1 and TG2 mouse hearts. (E) Assessment of fractional shortening percentage from NTG and transgenic lines. (F) Left ventricular end systolic dimension from NTG and transgenic lines. (G) Left

ventricular end diastolic dimension from NTG and transgenic lines. All results shown are the mean of eight independent experiments. Error bars indicate s.e.m. * $P < 0.05$ Vs. NTG.

Figure 3.

PiC gene upregulation does not affect Ca^{2+} -induced MPT. (A) Respiratory control assessment of isolated mitochondria from NTG, TG1 and TG2 mice using complex I- and complex II- dependent substrates. (B) Steady state ATP levels in NTG, TG1 and TG2 cardiac mitochondria. (C) Ca^{2+} -induced swelling under de-energized conditions of NTG, TG1 and TG2 cardiac mitochondria. For every condition, sensitivity to $1\mu\text{M}$ CsA was tested and completely inhibited swelling (not shown). (D) Representative traces of Ca^{2+} -retention capacity measurements of NTG, TG1 and TG2 cardiac mitochondria, with or without $1\mu\text{M}$

CsA. (E) Quantification of Ca^{2+} -retention capacity measurements in NTG, TG1 and TG2 cardiac mitochondria. All results shown are the mean of at least four independent experiments. Error bars indicate s.e.m.

Figure 4.

Cardiac-specific co-overexpression of PiC and CypD does not affect the Ca²⁺-induced MPT pore in isolated mitochondria. (A) Western blot showing enhanced expression of PiC in mitochondria from PiC TG1 mice, CypD in the CypD TG line and both proteins in the double transgenic (DTG) mice. (B) Representative images of hearts excised from the indicated NTG and transgenic lines. (C) Gravimetric assessment of NTG, TG1, CypD TG and DTG mouse hearts. (D) Ca²⁺-induced swelling under de-energized conditions of NTG and DTG cardiac mitochondria. For every condition, sensitivity to 1μM CsA was tested and

completely inhibited swelling (not shown). (E) Quantification of Ca^{2+} -retention capacity measurements of NTG and DTG cardiac mitochondria, with or without 1 or 5 μM CsA. All results shown are the mean of eight independent experiments. Error bars indicate s.e.m. * $P < 0.05$ Vs. NTG.

Figure 5.

Generation of a mouse strain with cardiac-specific PiC silencing. (A) Western blot showing decreased expression of PiC in mitochondria from tTA+shTG mice but not ANT, CypD, ATP synthase (AtpS) and VDAC as compared to its NTG, tTA and shTG counterparts. (B) Quantification of PiC protein levels as assessed by standard densitometry. (C) Representative images of hearts excised from the indicated NTG and tTA+shTG lines. (D) Heart Gravimetric assessment of NTG and tTA+shTG mouse hearts. (E) Assessment of fractional shortening percentage from the NTG and tTA+shTG lines. (F) Left ventricular

end systolic dimension from the NTG and tTA+shTG lines. (G) Left ventricular end diastolic dimension from the NTG and tTA+shTG lines. All results shown are the mean of at least four independent experiments. Error bars indicate s.e.m. * $P < 0.05$ Vs. NTG.

Figure 6.

PiC gene silencing does not affect Ca²⁺-induced MPT. (A) Respiratory control assessment of isolated mitochondria from NTG and tTA+shTG mice using complex I- and complex II-dependent substrates. (B) Steady state ATP levels in NTG and tTA+shTG cardiac mitochondria. (C) Ca²⁺-induced swelling under de-energized conditions of NTG and tTA+shTG cardiac mitochondria. For every condition, sensitivity to 1 μ M CsA was tested and completely inhibited swelling (not shown). (D) Representative traces of Ca²⁺-retention capacity measurements of NTG and tTA+shTG cardiac mitochondria, with or without 1 μ M

CsA. (E) Quantification of Ca^{2+} -retention capacity measurements of NTG and tTA+shTG mitochondria. (F) Representative traces of Ca^{2+} -retention capacity measurements in permeabilized adult cardiomyocytes from NTG and tTA+shTG mice. (G) Quantification of Ca^{2+} -retention capacity measurements of the permeabilized NTG and tTA+shTG cardiomyocytes. All results shown are the mean of at least four independent experiments. Error bars indicate s.e.m. * $P < 0.05$ Vs. NTG.