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Properties of the Permeability Transition Pore in Mitochondria Devoid of Cyclophilin D*

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Emy Basso‡§, Lisa Fante‡, Jonathan Fowlkes§, Valeria Petronilli‡, Michael A. Forte§¶, and Paolo Bernardi‡‖

From the ‡Department of Biomedical Sciences and Consiglio Nazionale delle Ricerche Institute of Neuroscience, University of Padova, Viale Giuseppe Colombo 3, I-35121 Padova, Italy and the §Vollum Institute, Oregon Health and Sciences University, Portland, Oregon 97239

We have studied the properties of the permeability transition pore (PTP) in mitochondria from the liver of mice where the *Ppif* gene encoding for mitochondrial Cyclophilin D (CyP-D) had been inactivated. Mitochondria from *Ppif*$^{-/-}$ mice had no CyP-D and displayed a striking desensitization of the PTP to Ca^{2+}, in that pore opening required about twice the Ca^{2+} load necessary to open the pore in strain-matched, wild-type mitochondria. Mitochondria lacking CyP-D were insensitive to Cyclosporin A (CsA), which increased the Ca^{2+} retention capacity only in mitochondria from wild-type mice. The PTP response to ubiquinone 0, depolarization, pH, adenine nucleotides, and thiol oxidants was similar in mitochondria from wild-type and *Ppif*$^{-/-}$ mice. These experiments demonstrate that (i) the PTP can form and open in the absence of CyP-D, (ii) that CyP-D represents the target for PTP inhibition by CsA, and (iii) that CyP-D modulates the sensitivity of the PTP to Ca^{2+} but not its regulation by the proton electrochemical gradient, adenine nucleotides, and oxidative stress. These results have major implications for our current understanding of the PTP and its modulation *in vitro* and *in vivo*.

The "permeability transition" is a sudden increase of the inner mitochondrial membrane permeability to ions and solutes, which causes dissipation of $\Delta\psi_m$,[1] loss of mitochondrial ion homeostasis, impairment of ATP synthesis, and diffusion of solutes down their concentration gradient (1). *In vitro*, at least, this is followed by an osmotically obligatory water flux across the inner membrane with passive swelling, outer membrane rupture, and cytochrome *c* release (2). This complex phenomenon is due to opening of a regulated, high conductance channel of unknown molecular structure, the PTP. PTP opening requires matrix Ca^{2+}, and its open-closed transitions are affected by a striking number of agents that may converge on a set of control elements such as the $\Delta\psi_m$ (3), matrix pH (4), adenine nucleotides (1), and the redox potential (5). Interest in the permeability transition as an executioner mechanism of cell death through Ca^{2+} deregulation and ATP depletion dates to the early 1990s (6–11) and was rekindled by the discovery that release of intermembrane proteins such as apoptosis-inducing factor, cytochrome *c*, and Smac-Diablo is instrumental in the activation of the apoptosome. The ensuing caspase 9 activation may lead to activation of effector caspase 3 in the so called intrinsic (mitochondrial) pathway to apoptosis (12).

A fundamental discovery was the identification of CsA as a high affinity inhibitor of the PTP (13–15). The putative receptor for CsA is CyP-D, a matrix peptidyl-prolyl *cis-trans* isomerase that is inhibited by CsA in the same range of concentrations that inhibits the pore (16) through an effect that does not require calcineurin inhibition (17). Largely through the use of CsA key advances have been made in understanding the role of the PTP in several *ex vivo* and *in vivo* models of disease such as ischemia-reperfusion injury of the heart (18, 19), ischemic and traumatic brain injury (20–27), late stage amyotrophic lateral sclerosis (28), acetaminophen toxicity (29), muscular dystrophy caused by collagen VI deficiency (30), hepatocarcinogenesis by 2-acetylaminofluorene (31), and fulminant hepatitis mediated by TNFα or Fas (32–34). Evidence that CyP-D is a modulator of the PTP remains indirect, however, and CyP-D overexpression did not cause the expected sensitization to cell death but rather protected from cell death induced by oxidative stress and mediated by mitochondria (35). To unambiguously resolve basic questions related to the role of CyP-D in PTP regulation, and the function of the permeability transition in normal biological processes like programmed and accidental cell death, we have generated a mouse line in which the expression of CyP-D has been eliminated by "knock-out" of the *Ppif* gene. We report here for the first time the properties of the permeability transition in mitochondria from *Ppif*$^{-/-}$ animals, which are devoid of CyP-D. Part of these results have already been presented in abstract form (36).

EXPERIMENTAL PROCEDURES

Generation and Characterization of Ppif$^{-/-}$ *Mice*—To identify mouse homologs of human CyP-D gene, degenerate oligonucleotide primers representing sequences encoding the unique N terminus of this molecule (amino acid sequence (residues 30–47) obtained from the purified human protein) and the common C terminus (amino acids 200–208) were used to PCR-amplify sequences encoding this molecule from cDNA generated from mouse liver RNA. To identify and characterize the region of the mouse genome encoding CyP-D, a unique set of PCR probes was generated from full-length mouse cDNA and used to screen a mouse bacterial artificial chromosome genomic library prepared from 129Sv ES cells. Several overlapping bacterial artificial chromosome clones were identified and genomic regions encoding *Ppif* identified by restriction enzyme digestion, PCR analysis, and Southern blotting. Eventually, a 23.5-kb fragment of genomic sequence containing the *Ppif* gene was characterized.

To generate mice in which the expression of *Ppif* has been eliminated, ES cells were cultured using standard conditions, transfected by

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¶ To whom correspondence may be addressed: Vollum Inst., L474, Oregon Health and Sciences University, 3181 S.W. Sam Jackson Park Rd., Portland, OR 97239. Fax: 503-494-4976; E-mail: forte@ohsu.edu.

‖ To whom correspondence may be addressed. Fax: 39-049-827-6361; E-mail: bernardi@bio.unipd.it.

[1] The abbreviations used are: $\Delta\psi_m$, mitochondrial membrane potential; CsA, cyclosporin A; CRC, calcium retention capacity; CyP-D, cyclophilin D; Δp, proton electrochemical gradient; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MOPS, 4-morpholinepropanesulfonic acid; PTP, permeability transition pore; Ub0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (ubiquinone 0).

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electroporation with the targeting construct, transfectants selected with appropriate antibiotics (G418 and ganciclovir), and candidate ES cells screened by PCR analysis and Southern blotting for replacement of the endogenous *Ppif* gene with the targeting construct. Male chimeras were subsequently mated with black, non-agouti C57BL/6 female mice and offspring evaluated for germ line transmission. Several of the chimeric males were able to pass the *Ppif* knock-out gene to progeny as assessed by both PCR analysis and Southern blotting. F1 heterozygotes were then back-crossed for eight generations into a C57BL/6 genetic background and isogenic heterozygotes intercrossed to generate homozygous wild-type and $Ppif^{-/-}$ animals.

To assess the expression of CyP-D protein in mice of the indicated genotypes, mitochondria were prepared from liver, heart, and kidney by homogenization and differential centrifugation following published protocols. Mitochondrial proteins were then separated on 15% SDS-polyacrylamide gels, proteins transferred to nitrocellulose, and blots probed for CyP-D using an antibody generated to unique N-terminal epitope from Affinity Bioreagents (Golden, CO) or the antibody described by Lin and Lechleiter (35). An anti-voltage-dependent anion channel antibody kindly provided by Dr. William Craigen (Baylor College of Medicine) was used as a control for loading.

Assays on Isolated Mouse Liver Mitochondria—Mitochondria were isolated from the livers of wild-type and $Ppif^{-/-}$ C57BL/6 mice exactly as described previously for rat liver mitochondria (37) except that 10 ml of isolation medium per liver were used. The CRC of mitochondrial preparations was assessed fluorimetrically in the presence of the Ca^{2+} indicator Calcium Green-5N (Molecular Probes) with a PerkinElmer Life Sciences LS50B spectrofluorimeter exactly as described previously (38). Oxygen consumption was measured polarographically with a Clark oxygen electrode in a closed 2-ml vessel. Mitochondrial swelling was measured as the decrease of 90° light scattering at 540 nm in a PerkinElmer Life Sciences 650-40 spectrofluorimeter. All instruments were equipped with magnetic stirring and thermostatic control. The incubation conditions are specified in the figure legends.

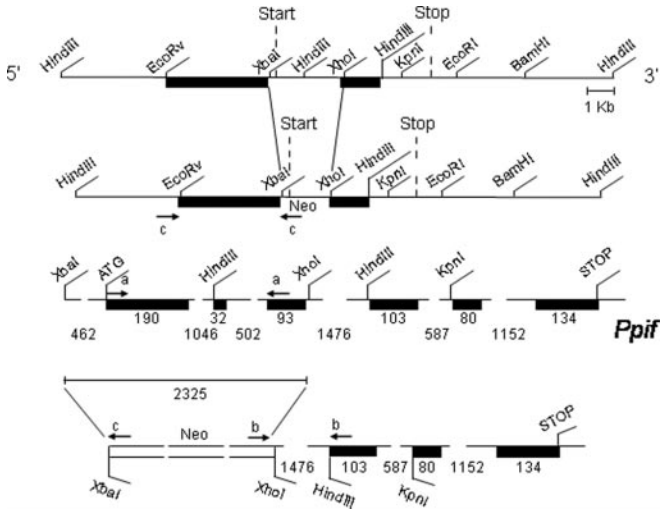

FIG. 1. **Structure of the wild-type *Ppif* gene and of the knock-out allele.** *Top line*, restriction map of the mouse genomic region containing the *Ppif* gene; regions of homology used for the targeted disruption are indicated by the *thick lines*. *Second line*, restriction map of the $Ppif^{-/-}$ alleles. *Third line*, expansion of the region of the mouse genome encoding *Ppif*; exons are indicated by *thick lines* and introns by *thin lines*, and the sizes of individual exons and introns are indicated. *Last line*, expansion of the regions of the mouse genome carrying the $Ppif^{-/-}$ allele in which the *neo* gene replaces the first three exons, including 20 bp upstream of the initiating ATG in the gene encoding CyP-D; positions of individual primer sets used for genotyping are indicated (see Fig. 2*A*).

RESULTS AND DISCUSSION

CyP-D (also known as CyP-F in mice) is a member of the larger cyclophilin family and is equivalent to the previously cloned cDNA encoding human CyP-3 (39). CyP-D is nuclearly encoded and contains a mitochondrial targeting presequence that is cleaved after translocation of the protein into the matrix (39). In addition, mature forms of the protein also contain a unique N terminus, which serves to identify CyP-D from other CyP isoforms. Probes generated from sequences encoding human CyP-D were used to identify cDNAs encoding murine CyP-D. Subsequently, sequences representing the *Ppif* gene encoding CyP-D were characterized (see "Experimental Procedures") and included 11 kb upstream and 6.5 kb downstream of the translational start and stop sites (Fig. 1). The *Ppif* gene itself encompasses 5.5 kb of genomic DNA and consists of six protein-coding exons separated by five introns (Fig. 1). Scans of complete human and mouse genome data bases indicate that a single gene encodes CyP-D in mammals and that the murine *Ppif* gene is located on chromsome 14.

To generate strains of mice deficient in CyP-D, a targeting plasmid was generated in which the *neo* gene was positioned so that the first three exons of the *Ppif* gene, including 20 bp upstream of the initiating ATG, were eliminated (Fig. 1). The targeting vector itself contained 7 kb of *Ppif* homology upstream of the initiating ATG and 2 kb of genomic sequence within the *Ppif* gene flanking the *neo* gene. The *neo* gene itself was positioned so that its transcription occurred from the strand opposite to that used to code for *Ppif*. ES cell lines in which the *Ppif* gene had been disrupted were identified by PCR analysis of genomic DNA and Southern blotting (data not shown). Positive ES cells were then injected into blastocysts and chimeric offspring evaluated for the ability to generate $Ppif^{+/-}$ offspring. F1 heterozygotes carrying the targeted allele were back-crossed eight times to C57BL/6 animals and heterozygotes then intercrossed to generate isogenic wild-type and $Ppif^{-/-}$ mice.

PCR analysis of genomic DNA from $Ppif^{+/+}$, $Ppif^{+/-}$, and $Ppif^{-/-}$ mice confirmed that the *Ppif* gene had been disrupted (Fig. 2*A*, *upper panel*). Analysis of CyP-D at the protein level revealed that expression was reduced to roughly 50% in liver mitochondria from $Ppif^{+/-}$ animals and that no CyP-D protein was detectable in mitochondria prepared from liver (Fig. 2*A*, *lower panel*), kidney, or heart (data not shown) of $Ppif^{-/-}$ mice. Despite the absence of CyP-D, mitochondria from $Ppif^{-/-}$ mice displayed basal, ADP- and uncoupler-stimulated rates of respiration that were indistinguishable from those of mitochondria prepared from wild-type mice (Fig. 2*B*), suggesting that CyP-D does not grossly affect energy conservation and ATP synthesis.

We then tested the properties of the permeability transition in liver mitochondria from wild-type and $Ppif^{-/-}$ mice with the sensitive CRC test, which measures the threshold Ca^{2+} required to open the PTP in a population of mitochondria in suspension. The experiments of Fig. 2*C* show that mitochondria from $Ppif^{-/-}$ mice required about twice the amount of Ca^{2+} necessary to open the PTP in wild-type mitochondria (compare *traces a* and *a′*). Addition of CsA caused the expected increase of CRC in wild-type (*trace b*) but not in $Ppif^{-/-}$ mitochondria (*trace b′*), while both populations were equally sensitive to Ub0 (compare *traces c* and *c′*) (see Fig. 4 statistical analysis). These experiments prove that CyP-D modulates the affinity of the PTP for Ca^{2+} and represent the first direct demonstration that CyP-D is the target for the inhibitory effects of CsA on the PTP.

The PTP is modulated by the Δp, in the sense that the pore open probability increases upon depolarization (*i.e.* as the membrane potential becomes less negative inside) and decreases at acidic matrix pH values (3). To assess whether the lack of CyP-D affected the PTP voltage dependence, we tested the effect of the addition of uncouplers to energized mitochondria preloaded with a small amount of Ca^{2+} that is not sufficient to open the PTP *per se* but is permissive for the subsequent opening by depolarization. Like mitochondria from wild-type animals, mitochondria from $Ppif^{-/-}$ mice readily opened

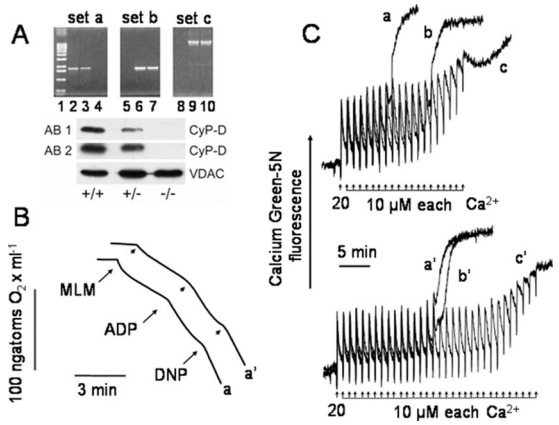

FIG. 2. **Respiratory activity and CRC of wild-type and *Ppif*$^{-/-}$ liver mitochondria: effect of CsA and Ub0.** *A*: *upper panel*, PCR products generated from genomic DNA prepared from wildtype *Ppif*$^{+/+}$ (*lanes 2*, *5*, and *8*), *Ppif*$^{+/-}$ (*lanes 3*, *6*, and *9*) and *Ppif*$^{-/-}$ mice (*lanes 4*, *7*, and *10*) were separated by agarose gel electrophoresis together with a set of size markers (*lane 1*) and probed using primer *sets a*, *b*, or *c* as indicated (see Fig. 1 for primer positions); *lower panel*, Western blots of total mitochondrial extracts prepared from mouse livers of the indicated *Ppif* genotypes; blots were probed with antibodies specific for CyP-D, AB-1, antibody from Affinity Bioreagents, AB-2, antibody described by Lin and Lechleiter (35); the blots were also probed with an anti-voltage-dependent anion channel antibody as a loading control (see "Experimental Procedures"). *B* and *C*, the incubation medium contained 250 mM sucrose, 10 mM Tris-MOPS, 5 mM glutamate-Tris, 2.5 mM malate-Tris, 1 mM P_i-Tris, and 20 μM EGTA-Tris. In the experiments of *C* only, 1 μM Calcium Green-5N was also added. Final volume: 2 ml, pH 7.4, 25 °C. *B*, where indicated 4 mg of wild-type (*trace a*) or *Ppif*$^{-/-}$ mitochondria (*trace a′*) were added (*MLM*), followed by 100 μM ADP and 50 μM 2,4-dinitrophenol (*DNP*) (*arrows*); *traces* shown are representative of four experiments. *C*, the experiments were started by the addition of 1 mg of wild-type (*traces a–c*) or *Ppif*$^{-/-}$ mitochondria (*traces a′–c′*) (addition not shown) followed 1 min later by the indicated concentrations of Ca^{2+} (*arrows*); in the experiments of *traces b* and *b′*, 1.6 μM CsA were added before mitochondria, and in those of *traces c* and *c′*, 20 μM Ub0 were added before mitochondria; *traces* shown are representative of results from 10 mitochondrial preparations of wild-type and *Ppif*$^{-/-}$ mitochondria for each condition.

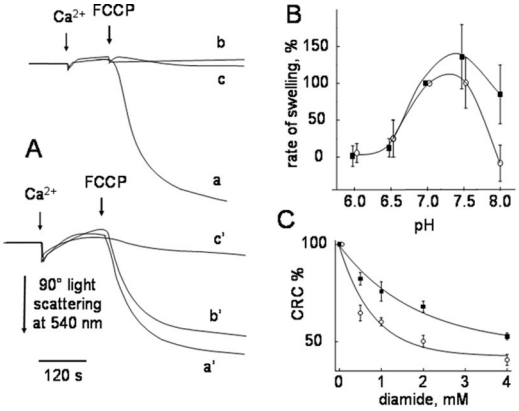

FIG. 3. **Effects of FCCP, pH, and diamide on wild-type and *Ppif*$^{-/-}$ liver mitochondria.** *A*, the incubation medium was the same as described for Fig. 2*B*. Final volume: 2 ml, pH 7.4, 25 °C. The experiments were started by the addition of 1 mg/ml wild-type (*traces a–c*) or *Ppif*$^{-/-}$ (*traces a′–c′*) mitochondria (data not shown). Where indicated (*arrows*) 50 μM Ca^{2+} (*traces a–c*) or 300 μM Ca^{2+} (*traces a′–c′*) and 200 nM FCCP (all *traces*) were added. In the experiments of *traces b* and *b′* 1 μM CsA were added before mitochondria, and in those of *traces c* and *c′* 20 μM Ub0 were added before mitochondria. *Traces* shown are representative of three experiments. *B*, the incubation medium contained 115 mM KSCN, 10 mM MOPS, 20 μM EGTA, and 2 μM rotenone. Medium pH was adjusted to 6.5, 7.0, 7.5, and 8.0 with KOH. The experiments were started by the addition of 1 mg/ml wild-type (*closed squares*) or *Ppif*$^{-/-}$ (*open circles*) mitochondria followed by 400 or 800 μM Ca^{2+}, respectively. Values on the *ordinate* refer to the rates of permeabilization measured after Ca^{2+} addition and are expressed as the percentage of the rates measured at pH 7.0. *C*, the experimental conditions were the same as described for Fig. 2*C*, except that the indicated concentrations of diamide were added immediately prior to mitochondria. Values on the *ordinate* were normalized to the CRC observed in the absence of diamide. For the experiments of *B* and *C error bars* represent the S.E. of four different preparations of mitochondria.

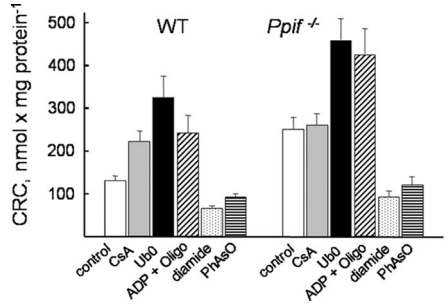

FIG. 4. **Effects of selected PTP inhibitors and inducers on the CRC of wild-type and *Ppif*$^{-/-}$ mitochondria.** The CRC was determined exactly as described for Fig. 2*C* for wild-type (*left set* of *bars*) and *Ppif*$^{-/-}$ mitochondria (*right set* of *bars*). Incubations were carried out in the absence of further additions (control: *open bars*, $n = 10$), in the presence of 1 μM CsA (*gray bars*, $n = 10$), of 20 μM Ub0 (*black bars*, $n = 10$), 100 μM ADP, and 1.26 μM oligomycin (*ADP + oligo*, *hatched bars*, $n = 6$), 4 mM diamide (*dotted bars*, $n = 6$), or 1 μM phenylarsine oxide (*PhAsO*, *striped bars*, $n = 3$). WT, wild-type. *Error bars* represent the S.E. of the number of replicate experiments indicated above.

the PTP upon the addition of FCCP (Fig. 3*A*, compare *traces a* and *a′*). At variance from the case of wild-type mitochondria, the PTP-dependent swelling response of *Ppif*$^{-/-}$ mitochondria was insensitive to CsA (compare *traces b* and *b′*), while it was as sensitive to Ub0 (compare *traces c* and *c′*). It should be stressed that no inhibitory effect was observed in *Ppif*$^{-/-}$ mitochondria even if the concentration of CsA was raised to 6.4 μM (results not shown).

The PTP open probability also displays a remarkable dependence on the pH of the incubation medium (1, 40). PTP inhibition occurs as pH is decreased from 7.4 to 6.4, and we have shown that the inhibitory effect is exerted from the matrix side of the inner membrane (3) through reversible protonation of histidyl residues (4). The PTP is also inhibited as the pH is increased above 7.4 through an undefined mechanism (4). To assess the dependence of PTP opening on matrix pH we used deenergized mitochondria incubated in a KSCN-based medium (4). In this system Ca^{2+} uptake is driven by the SCN^- diffusion potential (41), and Ca^{2+} accumulation occurs without changes of matrix pH, which under these conditions closely matches external pH (4). In these experiments, the PTP response was indistinguishable in *Ppif*$^{-/-}$ and wild-type mitochondria in the pH range 6.0–7.5, while the *Ppif*$^{-/-}$ mitochondria were somewhat more sensitive to inhibition by pH 8.0. These results indicate that CyP-D does not mediate the inhibitory effects of acidic matrix pH on the PTP and that the regulatory histidyl residue(s) that can be reversibly blocked by diethylpyrocarbonate (4) are not located on CyP-D.

The PTP is extremely sensitive to oxidative stress, and a dithiol-disulfide interconversion at vicinal dithiol(s) is of particular relevance in modulating the PTP response to depolarization. The PTP is sensitized as the couple is poised to a more oxidized state (5). Mitochondria from *Ppif*$^{-/-}$ mice were as sensitive as those from wild-type animals to the PTP-inducing effects of diamide (Fig. 3*C*), which acts through the redox-sensitive dithiol (5). If anything, *Ppif*$^{-/-}$ mitochondria appeared to require slightly lower concentrations of diamide, although the maximal effect was nearly identical to that observed in wild-type mitochondria (see also Fig. 4).

Quantitative assessment of the effects on the CRC already

described for CsA, Ub0, and diamide is presented in Fig. 4, which also documents the effects of ADP plus oligomycin, which inhibits the PTP (1), and of the vicinal dithiol cross-linker phenylarsine oxide, a potent PTP inducer that is effective even in the absence of added Ca^{2+} (3, 42). It can be seen that *Ppif*$^{-/-}$ mitochondria were fully sensitive to Ub0, which inhibits the pore through a specific site (43), and to ADP, which may be acting through a conformational effect on the adenine nucleotide translocator by locking it in the so-called m-conformation (2). The impression that Ub0 and ADP plus oligomycin are more potent inhibitors of the PTP in *Ppif*$^{-/-}$ than in wild-type mitochondria depends on their higher CRC. Indeed, if the basal CRC values are subtracted from those obtained in the presence of Ub0 or of ADP plus oligomycin the net effects of these treatments are not significantly different in the two groups of animals. Finally, Fig. 4 shows that both diamide and phenylarsine oxide equally affected the PTP in wild-type and *Ppif*$^{-/-}$ mitochondria.

In summary, our experiments demonstrate that the PTP can form and open in the absence of CyP-D. The lack of effects of CsA on the PTP in *Ppif*$^{-/-}$ mitochondria suggests that CyP-D represents the unique target for PTP inhibition by CsA. A second clear point emerging from our study is that CyP-D modulates the PTP sensitivity to Ca^{2+}. The Ca^{2+} resistance of the *Ppif*$^{-/-}$ mitochondria is similar to that observed in mitochondria from mice with genetic inactivation of the adenine nucleotide translocators (44), suggesting that the PTP sensitivity to Ca^{2+} may depend on multiple factors. It is important to stress that the PTP of *Ppif*$^{-/-}$ mitochondria maintains its basic regulatory features by the Δp and its sensitivity to activators other than Ca^{2+} and to inhibitors other than CsA.

Ppif$^{-/-}$ pups were born at the expected Mendelian ratio and were otherwise indistiguishable from wild-type, *Ppif*$^{+/+}$ C57BL/6 animals suggesting that CyP-D is dispensible for embryonic development and viability of adult mice. It is possible that this lack of an overt phenotype may be due to adaptive responses whereby the decreased sensitivity of the PTP to Ca^{2+} is bypassed by compensatory mechanisms that are not detectable in isolated mitochondria. An alternative explanation is offered by the finding that CyP-D overexpression desensitized cells from apoptotic stimuli, suggesting that CyP-D may also play a role as a cell survival-signaling molecule acting on one or more targets other than the PTP (35). This dual function of CyP-D suggests that the proapoptotic and antiapoptic functions may eventually balance in the *Ppif*$^{-/-}$ animals and that assessing the existence of specific phenotypes in *Ppif*$^{-/-}$ animals may require the thorough testing of *in vivo* disease models.

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