Journal of Bioenergetics and Biomembranes, Vol. 37, No. 3, June 2005 (© 2005)
DOI: 10.1007/s10863-005-6565-9

Genetic Dissection of the Permeability Transition Pore

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The permeability transition pore (PTP) regulates the structural re-organization of mitochondria in response to changes in cellular Ca^{2+} and is thought to be an important participant in mitochondrial responses to cell death signals. Although the proteins forming the PTP have yet to be rigorously identified, recent examination of the response of mitochondria, cells and tissues lacking putative components of the PTP have been reported. Studies on mitochondria lacking cyclophilin D (CyP-D) have proved that this protein is the target for PTP inhibition by CsA; yet they have also unequivocally demonstrated that the PTP can form and open in the absence of CyP-D. Likewise, studies in mice lacking the two adenine nucleotide translocators expressed in this species have shown that a functional PTP can form in the absence of these proteins. Thus, the inner mitochondrial membrane components of the PTP remain to be identified, and the absence of CyP-D may not preclude PTP opening in vivo – a finding that questions the conclusion that the PTP participates in cell death pathways only in response to a restricted set of challenges.

KEY WORDS: Permeability transition pore; cyclophilin D; adenine nucleotide translocator; cell death; apoptosis; necrosis; "knock-out" mice; calcium.

THE PERMEABILITY TRANSITION

Mitochondria play a pivotal role in cell survival and tissue development by virtue of their role in energy metabolism and apoptosis. These roles of mitochondria center, in part, around their participation in the dynamic processes by which cellular levels of Ca^{2+} are modulated. In this context, mitochondria play a key role in the regulation of cellular Ca^{2+} levels, accumulating cytoplasmic Ca^{2+} whenever the local cytoplasmic free Ca^{2+} rises above a critical "set point" and slowly releasing Ca^{2+} when cytoplasmic Ca^{2+} is lowered below this set point (for reviews, see Bernardi, 1999; Nicholls and Budd, 2000; Vandecasteele *et al*., 2001). In addition, the close coupling of mitochondrial and ER Ca^{2+} levels has defined an interaction that is tightly and subtly organized, and coordinated on a subcellular basis, so that spatial and temporal details of the Ca^{2+} signal may be defined by the spatial organization and possibly specialization of mitochondrial populations within cells (for review, see Rizzuto *et al*., 2004). Consequently, the mitochondrion's high capacity to store and release Ca^{2+} is likely to play a critical role in a variety of disease processes (e.g. Ichas *et al*., 1997) and in the control of programmed cell death (PCD). Here, mitochondria appear to be obligate participants through their role in the release of intermitochondrial membrane proteins like cytochrome *c* (cyt *c*), Smac-Diablo, AIF and endonuclease G, which trigger downstream events that include the formation of the "apoptosome," activation of downstream caspases which constitute the effectors of PCD, and nuclear degradation (for recent reviews, see Martinou and Green, 2001; Zamzami and Kroemer, 2001; van Loo *et al*., 2002).

Given this multifactorial role, Ca^{2+} homeostasis, metabolism and bioenergetics work as an integrated system. Therefore, regulation of ion fluxes across mitochondrial membranes, specifically the inner mitochondrial membrane (IMM), is essential since energy conservation in the form of a proton electrochemical potential difference ($\Delta\mu H$) is used to drive each process (for review, see

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Bernardi *et al*., 1999). The inner membrane possesses an intrinsically low permeability to ions and solutes, and a set of channels and transporters regulate mitochondrial ion fluxes and volume homeostasis. However, mitochondria in vitro can easily undergo a permeability increase to solutes with molecular masses of about 1,500 Da or lower (for reviews, see Zoratti and Szabo, 1995; Crompton *et al*., 2002; Halestrap *et al*., 2002). This permeability change, called the permeability transition (PT), has been encountered by biochemists for the last 50 years during mitochondrial preparation. In vitro at least, it is followed by an osmotically obligatory water flux across the membrane due to the high protein concentration of the mitochondrial matrix, resulting in passive swelling that is routinely monitored as a decrease in absorbance assessed spectrophotometrically. Under these in vitro conditions, the PT has dramatic consequences on mitochondrial function, resulting in the collapse of the membrane potential across the inner mitochondrial membrane required to drive the synthesis of ATP by the F0/F1 ATPase, and in the depletion of pyridine nucleotides and respiratory substrates, which eventually causes respiratory inhibition after transient uncoupling. Although common PT assays in vitro are based on swelling, swelling is not a necessary consequence of pore opening. Swelling results from the solute and water flux from the intermembrane/intercristal spaces to the matrix only if there is an osmotic imbalance. Indeed, in vitro, PT-dependent swelling can be completely prevented by tuning the concentration of non-diffusible macromolecules to counterbalance the osmotic pressure developed by the matrix proteins, conditions certainly more closely reflective of those encountered in living cells (Pfeiffer *et al*., 1995). Thus, and predictably for the needs of energy conservation, under normal conditions the inactive state of the PT would be favored, making prolonged opening an uncommon event. Rather, in intact cells, the PT may be active transiently (i.e., reversibly) to states that are not associated with mitochondrial swelling or stable changes of the $\Delta\mu$H. These transient states were originally noted at the single channel level as a flickering of the "mitochondrial megachannel" in the millisecond time scale between lower conductance open and closed states (Szabo and Zoratti, 1991). Transient activation of the PT has also been observed in isolated mitochondria and in whole cells and is likely to mediate the fast release of Ca^{2+} from mitochondria (Ichas *et al*., 1997; Huser *et al*., 1998; Petronilli *et al*., 1999, 2001).

In addition, recent attention has focused on the PT as a potential key participant in mitochondrial responses to apoptotic signals. In situ measurements with fluorescent probes during PCD showed changes of the mitochondrial membrane potential ($\Delta\psi_m$) consistent with PT-dependent mitochondrial depolarisation, giving rise to the idea that the PT represents a key element in determining the cell fate in PCD through release of cyt *c* and other apoptogenic proteins (for review, see Martinou and Green, 2001; Zamzami and Kroemer, 2001). However, the association of large amplitude swelling of mitochondria during PCD, in concert with mitochondrial depolarisation, is hotly debated; evidence for and against a requirement for mitochondrial swelling in apoptosis, and the role of BCL-2 family member in this process, exists in the literature (see Bernardi *et al*., 2001, for a recent review).

Several reports implicate transient activation of the PT, not associated with mitochondrial swelling, in processes that underlie PCD and action of BCL-2 family members, which control the integrity and response of mitochondria to apoptotic signals (De Giorgi *et al*., 2002). Furthermore, the ability of the PT to regulate, and respond to, mitochondrial Ca^{2+} plays a central role in these PCD pathways and in the mechanisms by which BCL-2 family members may influence this process (Ferrari *et al*., 2002; Pinton *et al*., 2002; Scorrano and Korsmeyer, 2003). For example, in cardiac myotubes exposed to apoptotic agents, Ca^{2+} spikes initiate depolarisation of mitochondria in discrete subcellular regions; these mitochondria initiate slow waves of depolarisation and Ca^{2+} release propagating through the cell. Travelling mitochondrial waves are prevented by anti-apoptotic BCL-2 family members, perhaps by reducing the transfer of ER Ca^{2+} stores to mitochondria, and are likely to involve PT activation, since they can be blocked by compounds like cyclosporin A (CsA) known to inhibit the PT (Pacher and Hajnoczky, 2001). Mitochondrial Ca^{2+} waves result in cyt *c* release, caspase activation and PCD. Mitochondrial Ca^{2+} release through the PT is critical for wave propagation and is promoted by the action of pro-apoptotic members of the BCL-2 family, which transform mitochondria into an excitable state by sensitising the PT to Ca^{2+} (Pacher and Hajnoczky, 2001; Csordas *et al*., 2002).

Additionally, the PT has been implicated in the structural reorganization of the mitochondria following activation of pro-apoptotic BCL-2 family members to ensure that cyt *c* release from mitochondria during PCD is rapid and complete (Scorrano *et al*., 2002). These studies have demonstrated that pro-apoptotic members of the BCL-2 family drive a striking remodelling of mitochondrial structure. During this process, individual cristae become fused and the junctions between cristae and the inner mitochondrial membrane (IMM) are opened, resulting in the mobilization of cyt *c* stores normally restricted to tubular cristae. This process may be independent of the action of pro-apoptotic BCL-2 family members but is inhibited by CsA, directly implicating the PT in this reorganization

(Scorrano *et al*., 2002). Thus, it appears that the apoptotic pathway bifurcates following the activation of pro-apoptotic BCL-2 family members, resulting in an initial release of 10–15% of the cytochrome *c* residing in the intermitochondrial membrane space (IMS) (Bernardi and Azzone, 1981) and consequent caspase activation, while a separate path of mitochondrial remodelling dependent on the PT may eventually lead to matrix swelling with complete release of cyt *c* and onset of mitochondrial dysfunction.

THE PT PORE

The PT occurs by the opening of an inner membrane channel, the mitochondrial permeability transition pore (PTP). A great deal of information is available about the functional properties of the PTP (see Bernardi *et al*., 1994; Zoratti and Szabo, 1995; for reviews, see Bernardi, 1999). The PTP can be viewed as a voltage-dependent channel; voltage dependence is an intrinsic property of the pore and has been demonstrated in isolated mitochondria with depolarizing H^+ or K^+ currents. Furthermore, a high conductance channel ($\sim$1 nS; the "mitochondrial megachannel") with the expected voltage-dependence has been demonstrated by electrophysiological approaches to correspond to the activity of the PTP. Ca^{2+} is the single most important factor for PTP opening in vitro, while the PTP open-closed transitions are also modulated by a large variety of physiological factors and drugs. Importantly, cell signaling may modulate PTP opening by affecting BCL-2 proteins (the PTP is inhibited by high BCL-2/Bax ratios), and the levels of lipid mediators such as ceramide (Novgorodov *et al*., 2005) and arachidonic acid (Penzo *et al*., 2004); (for reviews, see Bernardi *et al*., 2002; Tomassini and Testi, 2002).

Despite detailed functional characterization, the molecular components forming the PTP have remained elusive. On the basis of pharmacological studies, the PTP is believed to be due to the formation of dynamic multiprotein complexes at inner/outer membrane contact sites (for review, see Halestrap *et al*., 2000; Halestrap and Brennerb, 2003; Palmieri, 2004). These complexes are widely believed to involve the IMM adenine nucleotide translocase (ANT) (e.g., Halestrap *et al*., 1997a), in association with the outer mitochondrial membrane (OMM) voltage-dependent anion channel (VDAC) (Crompton *et al*., 1998) and a regulatory matrix protein, cyclophilin D (CyP-D), which binds in vitro to complexes of the VDAC and the ANT and constitutes the putative target for CsA inhibition of pore activity. In addition, the PTP may be regulated by association with hexokinase and the peripheral benzodiazepine receptor (e.g., McEnery, 1992; Beutner *et al*., 1996; for review see Zoratti and Szabo, 1995). However, the fact that the molecular target for most of the compounds reported to inhibit or induce PTP are not known, together with their poor specificity, has hampered progress in elucidating PTP structure and pathophysiological relevance.

Yet, key advances in our understanding of the physiological role of the PT have been primarily based in the fact the PTP can be inhibited by CsA (Crompton *et al*., 1988; Broekemeier *et al*., 1989). Indeed, largely through the use of CsA, major progress has 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 (Griffiths and Halestrap, 1993; Di Lisa *et al*., 2001), ischemic and traumatic brain injury (Folbergrova *et al*., 1997; Li *et al*., 1997; Friberg *et al*., 1998; Ferrand-Drake *et al*., 1999; Matsumoto *et al*., 1999; Okonkwo *et al*., 1999; Okonkwo and Povlishock, 1999; Yoshimoto and Siesjo, 1999), late-stage amyotrophic lateral sclerosis (Keep *et al*., 2001), acetaminophen toxicity (Haouzi *et al*., 2002), muscular dystrophy caused by collagen VI deficiency (Irwin *et al*., 2003), hepatocarcinogenesis by 2-acetylaminofluorene (Klohn *et al*., 2003), and fulminant hepatitis mediated by TNFα and Fas (Feldmann *et al*., 2000; Crouser *et al*., 2002; Soriano *et al*., 2004).

PTP inhibition by CsA was first observed in isolated mitochondria and prompted a number of studies aimed at identifying the mitochondrial target(s) for CsA and the mechanism for inhibition of the PT. An early suggestion was that a mitochondrial matrix cyclophilin (CyP) represents the target for CsA, and therefore for PT inhibition (Halestrap and Davidson, 1990). An 18 kDa CyP isoform, CyP-D, exclusively found in mitochondria was later identified (Connern and Halestrap, 1992; Woodfield *et al*., 1997). Initial evidence supporting the idea that CyP-D played a direct role in the PTP emerged from an examination of the $K_{0.5}$ values of CsA analogs as inhibitors of its PPIase activity, which were essentially identical to those required for inhibition of the PTP. In addition, the number of sites to which CsA must bind to give 100% inhibition of the PTP corresponds to the concentration of PPIase within the matrix (Griffiths and Halestrap, 1991; Connern and Halestrap, 1992). Moreover, the "mitochondrial megachannel" observed by electrophysiological approaches is also inhibited by CsA (Szabo and Zoratti, 1991). Finally, purification of mitochondrial CsA binding proteins from total mitochondrial extracts by CsA affinity chromatography demonstrated that only CyP-D could be specifically eluted and that modulation of CyP-D binding to inside-out submitochondrial particles matched the predictions based on properties of PTP modulation (Nicolli *et al*., 1996).

At variance from immunosuppression, inhibition of the PTP by the CsA/CyP-D complex does not involve inhibition of calcineurin, an additional target inhibited by CsA. Indeed, the PTP can be inhibited by non-immunosuppressive derivatives of CsA (Nicolli *et al*., 1996). Thus, opening of the PTP is inhibited by CsA and some of its analogs, such as *N*-methylvaline-4-CsA (MeValCsA), a compound that has no or very limited immunosuppressive effect, but not by the immunosuppressor and calcineurin inhibitor FK506, which does not bind CyPs (Halestrap *et al*., 1997b). However, it should be stressed that CsA is not a true pore blocker; inhibition is transient in longer time-frame experiments (Broekemeier and Pfeiffer, 1995) and can be largely relieved by increasing matrix Ca^{2+} (Bernardi *et al*., 1993). Thus, CyP-D is likely a key regulator of the activity of the PTP, rather than a core component of the proteinaceous pore. Consistent with this idea, some studies indicate that during conditions of Ca^{2+} overload and oxidative stress CyP-D shifts from the matrix to the inner membrane, where it should interact with the PTP (Connern and Halestrap, 1994). These data support the idea that CsA inhibits the PTP through modulation of CyP-D binding to core components of the PTP, and establish CyP-D as a key regulator of PTP function. However, evidence that CyP-D is a modulator of the PTP remains indirect; and CyP-D overexpression did not cause the expected sensitization but rather protected cells from death induced by oxidative stress and mediated by mitochondria (Lin and Lechleiter, 2002).

GENETIC ANALYSIS OF THE ROLE OF THE ANT AND CYP-D IN PTP FORMATION

Over the past two years, the use of homologous recombination in mice to generate cells, tissues and animals in which the expression of putative key components of the PTP have been eliminated has allowed critical tests of the role of some of these proteins in the formation of the PTP and its physiological role. These genetic studies have focused on the ANT and CyP-D.

The PTP in Mitochondria Lacking the ANT

Nearly all essential metabolic pathways in a variety of tissues require an abundant supply of cytosolic ATP, produced in large part by mitochondrial oxidative phosphorylation. ATP synthesized in the matrix is then exported to the cytosol in exchange for incoming ADP by the ANT. The ANT is the most abundant member of a family of IMM transporters that have a conserved structure, and a variety of in vitro studies have shown that members of this family, under specific conditions, can form channel-like structures (Dierks *et al*., 1990; Brustovetsky *et al*., 2002). In addition, much biochemical evidence suggests that a CsA-inhibitable, PTP-like complex can be formed from purified ANT and CyP-D. For example, reports have described a CsA-sensitive permeabilization of liposomes reconstituted with CyP-D together with the ANT and VDAC (Crompton *et al*., 1998). In contrast, others have recorded a high conductance "mitochondrial megachannel" in yeast mitochondria from cells in which the expression of all ANT isoforms had been eliminated (Lohret *et al*., 1996). Thus, the involvement of the ANT in the PTP has lacked univocal documentation.

In order to directly test the involvement of the ANT in PTP formation and role of the PTP in apoptotic processes, Kokoszka *et al*. (2004) have used genetic strategies available in mice to create animals in which the genes encoding the two isoforms of the ANT expressed in this species have been selectively eliminated in the liver. As expected, Western blot analysis demonstrates that neither ANT isoform is present in double-mutant livers. Mitochondria prepared from ANT-deficient livers exhibited increase of respiration that is likely the result of the specific upregulation of cytochrome oxidase. Somewhat surprisingly, ANT-deficient and control mitochondria were able to undergo a Ca^{2+}-dependent, CsA-inhibitable PT, demonstrating that the ANT is not required to form a functional PTP. More predictably, the absence of the ANT resulted in the selective loss of regulation of the PTP by ANT ligands like atractyloside (which increases the probability of pore opening) and ADP (which increases the probability of pore closure). The PTP in ANT-deficient mitochondria required three-fold higher levels of Ca^{2+} for activation when compared to control mitochondria, suggesting that the ANT has an essential role in regulating the PT by modulating the sensitivity of the PT to Ca^{2+} activation and ANT ligands. Finally, hepatocytes prepared from control and ANT-deficient livers showed identical responses to activation of receptor-mediated apoptotic pathways initiated by TNF$\alpha$and Fas application, demonstrating that the ANT is not required in receptor-activated apoptotic pathways.

The PTP in Mitochondria Lacking CyP-D

In order to unambiguously resolve basic questions related to the role of CyP-D in PTP regulation, and the function of the PT in normal biological processes, three groups have recently reported the generation of mice in which the gene encoding CyP-D (*Ppif*) has been eliminated, resulting in mitochondria devoid of this protein. In all studies, $Ppif^{-/-}$ animals were born at the expected Mendelian ratio, and were otherwise indistinguishable from wild-type,

Ppif$^{+/+}$ animals suggesting that CyP-D is dispensable for embryonic development and viability of adult mice.

Basso *et al*. (2005) carefully studied the properties of the PTP in mitochondria from the livers of CyP-D-null mitochondria, which as expected lacked CyP-D as assessed by Western blot analysis. Despite the absence of CyP-D, mitochondria displayed basal, ADP- and uncoupler-stimulated rates of respiration that were indistinguishable from those of mitochondria prepared from wild-type mice, suggesting that CyP-D does not grossly affect energy conservation and ATP synthesis, as also concluded in a similar study of Nakagawa *et al*. Examination of mitochondria lacking CyP-D demonstrated that the PTP can form and open in the absence of CyP-D, consistent with its role as a regulator but not as a core structural component of the PTP. Indeed, the PTP in Cyp-D-null mitochondria showed a striking desensitization to Ca^{2+} in that opening required about twice the Ca^{2+} load necessary to open the pore in strain-matched, wild-type mitochondria. On the other hand, the PTP response to ubiquinone 0, depolarization, pH, adenine nucleotides and oxidative stress was indistinguishable in mitochondria from wild-type and *Ppif*$^{-/-}$ mice, indicating that none of these key regulators of the pore is modulated by CyP-D.

These general conclusions are corroborated by studies from two other groups that have generated *Ppif*$^{-/-}$ mice (Baines *et al*., 2005; Nakagawa *et al*., 2005). Specifically, these studies also document the ability of CyP-D-null mitochondria to accumulate higher levels of Ca^{2+} prior to PTP opening, and the presence of a CsA-insensitive PT at these higher concentrations of Ca^{2+} that mimics the characteristics of the PT of wild-type mitochondria treated with CsA.

Since the regulation of the PT by members of the BCL-2 family of proteins has been the subject of some debate, these latter groups also tested the release of cyt *c* in vitro from mitochondria prepared from the liver of wild-type and *Ppif*$^{-/-}$ mice following the application of recombinant forms of pro-apoptotic BCL-2 proteins. Treatment of CyP-D-null mitochondria with either tBID or BAX demonstrated that CyP-D deficiency had no effect on the ability of these proteins to promote the release of cyt *c* when compared to wild-type mitochondria. Furthermore, Baines and colleagues show that CyP-D deficient cells are not resistant to apoptosis otherwise caused by overexpression of BAX. In contrast, Ca^{2+}-induced (i.e., PTP-dependent) cyt *c* release was predictably reduced in CyP-D deficient mitochondria at Ca^{2+} loads that caused pore opening only in the wild-type mitochondria.

While it should not be surprising that matrix CyP-D does not play a role in cyt *c* release by tBID and BAX added to isolated mitochondria, a protocol that directly permeabilizes the outer mitochondrial membrane, these results have been used to conclude that the PT may only play a role in specific kinds of cell death, perhaps those resulting primarily in necrotic rather than apoptotic responses. In this context, several key points seem relevant. First, studies in rats have clearly shown that CsA treatment is able to potently inhibit the hepatotoxic effects of TNF-α, a pathway requiring the action of pro-apoptotic BCL-2 proteins, by desensitizing the mitochondrial proapoptotic pathway through PTP inhibition (Soriano *et al*., 2004). Second, the studies outlined above demonstrate that cells lacking CyP-D, and the ANT, can still undergo the PT, although the PT in the absence of these proteins shows an altered regulation. Third, overexpression of CyP-D should serve to sensitize cells to apoptotic stimuli if the PT were involved in these cellular responses. Baines *et al*. (see below) demonstrate that overexpression of CyP-D in the hearts of transgenic animals results in phenotypes consistent with the activation of apoptotic processes in these cells. Finally, it remains to be established whether the absence of CyP-D affects cristae remodelling after tBID and BAX translocation to mitochondria in situ.

Baines *et al*. and Nakagawa *et al*. also examined the responses of cells [embryonic fibroblasts (MEFs), thymocytes, and hepatocytes] prepared from *Ppif*$^{-/-}$ mice to agents promoting apoptosis. Cells missing CyP-D show increased resistance to oxidative stress (as induced by H_2O_2) and increased cytoplasmic Ca^{2+} (as mediated by A23187, ionomycyin or thapsigargin). In the studies of Baines et al., the resistance of CyP-D-null cells to H_2O_2 was reversed on re-expression of CyP-D but not by re-expression of mutant forms of CyP-D lacking PPIase activity. However, no difference was observed between wild-type or CyP-D-deficient cells and tissues following the applications of agents (staurosporine, TNF-α, X-irradiation) that activate apoptotic pathways dependent on pro-apoptotic members of the BCL-2 family. These results support conclusions generated in the in vitro studies described above suggesting that the PT may only participate in the response of cells to a subset of agents that induced cell death. In each of these studies, cell death is assessed by a variety of approaches (Annexin-V staining, TUNEL assay, Cell-Titer Blue assay, propidium idodide staining). Nakagawa and colleagues suggest that cell death mediated by reactive oxygen species and Ca^{2+} overload is primarily necrotic rather than apoptotic, since cell death in these cases is not associated with caspase activation, is not affected by caspase inhibition, is characterized by an early disruption of the plasma membrane and is not associated with nucleosomal fragmentation of genomic DNA. In addition, both reports document that cardiac ischemia/reperfusion injury, which results in the generation

of reactive oxygen species and Ca^{2+} overload, irrespective of the mouse genotype, causes less damage in *Ppif*$^{-/-}$ animals in a response that is similar to that observed following treatment of wild-type animals with CsA.

Finally, much has also been made of the fact that animals devoid of CyP-D develop normally and appear to lead normal, healthy lives (Halestrap, 2005). One plausible possibility is that this lack of an overt phenotype may be due to adaptive responses. For example, animals devoid of many of the key members of the BCL-2 family [BID (Yin *et al*., 1999), BAD (Ranger *et al*., 2003), BAK (Lindsten *et al*., 2000), BCL-2 (Veis *et al*., 1993), BAX (Knudson *et al*., 1995)] complete normal development and, in a number of instances, only display phenotypes following long term growth (Zinkel *et al*., 2003) or when challenged (Lindsten *et al*., 2000). Indeed, Baines *et al*. (2005) and Nakagawa *et al*. (2005) both demonstrate that hearts from CyP-D-null mice are resistant ischemia/reperfusion injury. Future studies on CyP-D-null mice will almost certainly be based in a careful examination of the response of cells and tissues to specific forms of apoptotic challenge. Thus, although it may be that the PT is only involved in the response of cells to specific forms of cell death stimuli, the data available at this point does not permit definitive conclusions on the role of the PT in normal apoptotic responses, including those regulated by members of the BCL-2 family.

Overexpression of CyP-D

Since CyP-D is an activator of the PT, one might predict that gain-of-function studies based in the overexpression of this protein would lead to an increased sensitivity to agents promoting cell death. In contrast, a number of groups have demonstrated that overexpression of CyP-D in cultured cells leads to repression of apoptosis as induced by a wide variety of agents, but differ on whether the PPIase activity of this protein is required for this suppression (Lin and Lechleiter, 2002; Li *et al*., 2004; Schubert and Grimm, 2004). In addition, one study concluded that overexpression of CyP-D inhibited apoptosis but promoted necrosis (Li *et al*., 2004). Baines *et al*. examined this question in a whole animal setting by transgenic overexpression of CyP-D in the heart. In contrast to wild-type or CyP-D null mitochondria, heart mitochondria overexpressing CyP-D showed alterations in mitochondrial architecture and loss of cristae. Mitochondria isolated from the hearts of transgenic mice displayed pronounced swelling at baseline that was corrected by polyethylene glycol-induced shrinkage in a CsA-sensitive manner. Finally, overexpression of CyP-D in cardiac myocytes led to increased TUNEL staining of the heart, increased cyt *c* release, increased caspase 9 activation with a corresponding cardiac hypertrophy, and reduction in cardiac function over time, as might be expected if CyP-D promoted cell death through the PT.

CONCLUSIONS

The value of loss-of-function genetic analysis in determining the necessity and sufficiency of a particular gene product in specific biochemical pathways or phenotypic responses is well established, and represents an important complement to traditional biochemical analysis. Over the past two years, the application of this approach in mice has provided critical tests of the role of the ANT and of CyP-D in the formation/activity of the PTP.

While the results of Kokoszka *et al*. (2004) would seem to unequivocally demonstrate that the ANT is not required for PTP formation, this conclusion has been reported as "controversial" (Halestrap, 2004) for a number of reasons. The strongest argument is that a "low level" of ANT expression (below the levels detected by western blots and respirometry) could have been present in the liver, and would be responsible for the PT observed. We note that a striking feature of the work of Kokoszka *et al*. is that the PT was insensitive to opening by atractylate and to closure by ADP. Since these two compounds affect the PTP through the ANT, it is difficult to see how the putative ANT molecules present in mitochondria should not respond to atractylate and ADP, and yet be able to promote a CsA-sensitive PT. More plausible appears the possibility that another member of the mitochondrial carrier family can form "variant" PT pores in the absence of ANT. Under this scenario, the pore formed remains sensitive to CsA, implying the existence of additional targets for CyP-D regulation of the PTP other than the ANT; and the resulting "variant" pores exhibit a diminished Ca^{2+} sensitivity. The key question then becomes not whether the ANT is required for PT formation (which is clearly ruled out by the genetic studies of Kokoszka *et al*.) but rather what component(s) of the IMM is/are involved in the creation of the PTP under normal circumstances; a pore that can be regulated, either directly or indirectly, by the ANT and CyP-D. Hopefully, future genetic and biochemical studies will provide conclusive evidence aimed at resolving this issue.

A clear picture of the role of CyP-D in PTP modulation emerges from the work of Basso *et al*. (2005), Baines *et al*. (2005), and Nakagawa *et al*. (2005). All studies confirm that a PT can take place in mitochondria devoid of this protein, and that this PT is insensitive to inhibition by CsA. Thus, these studies clearly document that CyP-D

is the unique target for PTP inhibition by CsA. However, the PTP can still open when mitochondrial are challenged with higher Ca^{2+} concentrations, consistent with the role of CyP-D as a regulator, not a key structural component, of the PTP. Furthermore, the results presented by Basso *et al*. indicate that CyP-D represents the unique target for PTP inhibition by CsA, but does not participate in pore regulation by the proton electrochemical gradient, adenine nucleotides and oxidative stress. Therefore, since the PTP maintains its basic regulatory features, i.e. modulation by the Δp and sensitivity to activators and inhibitors other than CsA, the transition of the PTP to an unregulated state in the absence of CyP-D appears unlikely (He and Lemasters, 2002). It must also be stressed that, at variance from the conclusions of Baines *et al*. and of Nakagawa *et al*., the in vivo studies on *Ppif*$^{-/-}$ mice can only be interpreted to discuss the role of CyP-D, not of the PTP, on apoptosis and necrosis. In other words, the inference that a PT does not take place because CyP-D is absent has not been documented in vivo, while upregulation of death signalling pathways converging on the PTP in *Ppif*$^{-/-}$ mice, which can bypass the effect of the absence of CyP-D, remains an obvious possibility (Basso *et al*., 2005).

In conclusion, while the genetic studies outlined in this review have answered a number of questions related to modulation of the PTP by the ANT and CyP-D, the structural features of the PTP remain undefined, and key questions about its role in pathophysiology are still open. The availability of the genetic reagents outlined here will clearly aid the development of clear and conclusive answers as to the identity of key components of the PTP and the role of this complex in the response of cells and tissues to both normal and pathological stimuli.

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