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Review

Signal transduction to the permeability transition pore

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ABSTRACT

The permeability transition pore (PTP) is an inner mitochondrial membrane channel that has been thoroughly characterized functionally, yet remains an elusive molecular entity. The best characterized PTP-regulatory component, cyclophilin (CyP) D, is a matrix protein that favors pore opening. CyP inhibitors, CyP-D null animals, and in situ PTP readouts have established the role of PTP as an effector mechanism of cell death, and the growing definition of PTP signalling mechanisms. This review briefly covers the functional features of the PTP and the role played by its dysregulation in disease pathogenesis. Recent progress on PTP modulation by kinase/phosphatase signal transduction is discussed, with specific emphasis on hexokinase and on the Akt-ERK-GSK3 axis, which might modulate the PTP through CyP-D phosphorylation.

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1. General features of the permeability transition pore

The permeability transition pore (PTP) is a voltage- and Ca^{2+}-dependent, cyclosporin A (CsA)-sensitive, high conductance channel, whose opening leads to permeabilization of the inner mitochondrial membrane (IMM) to solutes with molecular masses up to 1500 Da. A prolonged PTP opening has major consequences on energy metabolism and cell viability. Mitochondria depolarize due to equilibration of the proton gradient and the initial uncoupling is followed by release of matrix pyridine nucleotides resulting in respiratory inhibition and generation of reactive oxygen species (ROS) via the direct transfer of electrons to molecular oxygen. Oxidative phosphorylation and ATP synthesis cease, and the F_0F_1 ATP synthase starts working in reverse, hydrolyzing ATP generated by glycolysis or by residual functional mitochondria. As a result, a bioenergetic failure rapidly occurs [1]. Moreover, ions and solutes with molecular mass below the pore size equilibrate across the IMM, inducing disruption of metabolic gradients and release of the Ca^{2+} stored in the matrix. The colloidal osmotic pressure exerted by the high protein concentration in the matrix causes its swelling. Inner membrane cristae unfold and eventually may disrupt the outer membrane, leading to release of intermembrane proteins, including pro-apoptotic factors [2]. Thus, PTP opening prompts the demise of the cell, either through apoptosis (if enough ATP is present to fuel caspase activity), or through necrosis (that follows loss of Ca^{2+} homeostasis and mitochondrial dysfunction). The mode of cell demise could be necrotic when the permeability transition (PT) occurs in a fraction of mitochondria sufficient to cause ATP depletion. A more limited number of permeabilized mitochondria would lead to release of proapoptotic factors, and ATP production by the residual healthy mitochondria would be enough to support apoptosis execution. In a cell, a subpopulation of mitochondria may have a lower threshold for opening (e.g. those spatially closer to the triggering signal) and therefore open the PTP first. Ca^{2+} or other diffusible signals such as superoxide sparks [3] will then be sensed by other mitochondria, spreading PTP opening to the surrounding

Abbreviations: Aβ, amyloid beta; ANT, adenine nucleotide translocator; CsA, cyclosporin A; CyP-D, cyclophilin D; ERK, extracellular signal regulated kinase; GPCRs, G protein coupled receptors; GSK3, glycogen synthase kinase 3; HK, hexokinase; Hsp90, heat shock protein 90; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; P_i, phosphate anion; PiC, phosphate carrier; PINK1, PTEN induced kinase 1; PKC, protein kinase C; PKG, protein kinase G; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B; PP2Cm, mitochondrial protein phosphatase 2C; PT, permeability transition; PTP, permeability transition pore; RISK, reperfusion injury salvage kinase; ROS, reactive oxygen species; RTKs, receptor tyrosine kinases; SHP-1, SH2-containing tyrosine phosphatase 1; TRAP-1, tumor necrosis factor receptor-associated protein 1; VDAC, voltage-dependent anion channel

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organelles. In highly specialized cell types, these waves of mitochondrial depolarization could have functional implications. For instance, mitochondria isolated from synaptosomes have a lower threshold for PTP opening than mitochondria from other regions of the neuron [4]. It is possible to envisage a scenario in which synaptic damage does not diffuse to the neuron soma. It is also possible that PTP is involved in the autophagic disposal of damaged or aging mitochondria, which would become more susceptible to pore opening [1].

Given the importance of the PTP for cell biology, it comes as no surprise that its open–closed transitions are strictly regulated by a number of effectors, including a wide variety of cell death regulators. The undecapeptide CsA desensitizes the pore, i.e. increases its opening threshold by binding the mitochondrial chaperone cyclophilin D (CyP-D) [5]. Experiments performed on isolated mitochondria have shown that an increase in the matrix Ca^{2+} content is a key permissive factor for most PTP inducers; Ca^{2+} effect can be competitively inhibited by other Me^{2+} ions, such as Mg^{2+}, Sr^{2+} and Mn^{2+} [6]. However, we have recently found that CyP-D masks an inhibitory site for phosphate anion (P_i), which is the actual PTP desensitizing agent [7]. In fact, when P_i was replaced by other anions, the sensitivity of the PTP to Ca^{2+} remained identical in naïve and CsA-treated wild type mitochondria, as well as in CyP-D null mitochondria. We postulate that CyP-D favors the PTP open conformation by making the P_i site on the pore not available. When CyP-D is absent or bound to CsA, P_i binding to the PTP lowers its open probability, given a sufficient free P_i concentration. As a consequence, PTP induction in cells would be modulated by local changes in P_i concentration. This could be particularly relevant for non-excitable cells; conversely, in cardiomyocytes or neurons oxidant stress mechanisms appear to be dominant factors responsible for PTP opening [8]. Accordingly, pro-oxidants molecules (like *tert*-butylhydroperoxide, diamide, phenylarsine oxide) are recognized pore inducers, whereas proteins involved in antioxidant defences (like catalase, superoxide dismutase and glutathione) act as pore desensitizers [6]. Proton concentration also regulates the PTP. The optimum for pore opening is observed at matrix pH 7.4, whereas the opening probability sharply decreases either by lowering or by increasing matrix pH [9]. The PTP is voltage-dependent, and a high (inside negative) membrane potential stabilizes it in the closed conformation. We have postulated the existence of a sensor that translates the changes of either the transmembrane voltage or the surface potential into changes of the PTP open probability [10].

2. The channel

These observations match data obtained studying the PTP by electrophysiological means in mitoplasts [11]. The channel was found to locate in the IMM or at residual contact sites between mitochondrial membranes, and displays a maximal conductance in the 0.9–1.3 nS range [11,12]. Conductance substates can be recorded. A characteristic half-conductance or hemichannel is compatible either with a dimeric structure, or with a channel capable of entering a conformation with half the maximal conductance. Rapid conversions among conductance substates of the channel (flickerings) could allow passage of protons or Ca^{2+} without equilibration of small molecular weight metabolites. These transient PTP openings would not be associated with irreversible mitochondrial changes, but with physiological roles of the PTP, such as regulation of protein import, of matrix volume and pH, cristae remodeling, redox equilibrium, and Ca^{2+} release [1,10]. Electrophysiological properties of the hemichannel also include Ca^{2+}-dependence, with high ($\geqslant$100 μM) Ca^{2+} needed to observe sustained activity, and anion-selectivity, with a ratio of permeability coefficients $P_{Cl}/P_K \approx 7$–18. However, for short periods and in rare cases the channel can display cation-selective conductances, and this selectivity was also observed in different experimental settings [12]. Also similarly to established PTP features, ATP or Mg^{2+} at acid pH and reducing agents are able to inhibit the channel. The pattern of voltage sensitivity is complex. The hemichannel is open in the absence of any transmembrane voltage, but often shows a tendency to close at positive or negative ($\leqslant$40 mV) voltages. Nonetheless, some channels promptly close at low (<40 mV) voltages, whereas others require prolonged application of higher potentials. These changes cannot depend on stochastic variations, as repeated applications on the same patch of voltage protocols always induce the same channel response [11,13,14]. It was therefore postulated that this variability in voltage-dependence is caused either by interactions with regulatory proteins or by post-translational modifications, possibly phosphorylation, of the voltage sensor. Accordingly, several lines of evidence point towards a role of phosphorylations in PTP modulation (see below).

Other channels could also affect PTP activity. Following its electrochemical gradient, K^+ enters the matrix through IMM channels, whose inhibition results in hyperpolarization, which in turn enhances ROS production by reducing respiratory chain components. As widely reported, ROS oxidize thiols and thus open the PTP. PTP opening downstream to transient mitochondrial hyperpolarization has been reported in several studies. Alternatively or in addition, ROS could open the pore following activation of kinase signalling pathway. According to this view, in mitoplasts hypoxia opens the mitochondrial BK-channel and inhibits the PTP [15], and inhibitors of this BK-channel or of a mitoKATP channel sensitize the pore, measured as CsA-sensitive mitochondrial swelling [15,16]. Recently, a voltage-dependent, Shaker-like K^+ channel, termed $K_V1.3$, has been characterized in the IMM. $K_V1.3$ is endowed with a rapid opening following depolarization, and its inhibition with selective toxins prompts ROS production and a CsA-sensitive mitochondrial depolarization, indicating PTP opening. The action of the toxins can be mimicked by a specific residue on the pro-apoptotic protein Bax, which can similarly block the channel triggering cytochrome *c* release and apoptosis [17]. This observation is particularly important, as it offers a possible functional link between apoptotic pathways governed by Bcl-2 family proteins and the PTP. However, the functional connection between K^+ channel regulation and PTP opening is controversial, and experimental results probably dependent on the context. A viral protein, the HTLV-1 p13, triggers an inward mitochondrial K^+ current. The ensuing depolarization induced by p13 was proposed to increase production of ROS by enhancing respiratory chain activity, eventually lowering the threshold for PTP opening [18].

3. The core of the pore: molecular structure of the PTP

The precise mechanisms of PTP regulation are only partially understood, as the molecular structure of the pore remains an unsolved riddle. The present consensus model postulates that the channel is originated by a supramolecular complex. Several layers of complexity render dissection of single components a difficult task. Assemblage of PTP components might be a rare event, to avoid unwanted mitochondrial depolarization and damage. It is also possible that, in particular cell types or following specific triggering conditions, different protein multimers assemble to form the PTP, possibly resulting in diverse sensitivities to modulators or in changes of conductance properties [8]. A subset of proteins was proposed to constitute core components of the channel, but rigorous genetic testing in the last few years has excluded this pos-

sibility for most of them. These candidate components included the outer mitochondrial membrane (OMM) porin, aka voltage-dependent anion channel (VDAC); the matrix chaperone CyP-D; the adenine nucleotide translocator (ANT) in the IMM; and more recently the mitochondrial phosphate carrier (PiC).

Evidence linking VDAC to the PTP included the observations that its activity is modulated by many factors that also affect the PTP, such as NADH, Ca^{2+} and glutamate. Disruption of the binding between hexokinase (HK) and VDAC onto mitochondria was reported to prompt PTP opening and cell death [19]. Accordingly, we have observed that HK displacement from mitochondria induces PTP opening and apoptosis, but this occurs even in the absence of any binding between HK and each of the three VDAC isoforms, whereas disassembly of the HK/VDAC complex could be observed in conditions that inhibit pore opening, i.e. following cell treatment with the CsA analogue Debio 025 [20]. In addition, the PTP of mitochondria prepared from VDAC1$^{-/-}$, VDAC3$^{-/-}$ and VDAC1/3$^{-/-}$ mice, or from fibroblasts lacking all three VDAC isoforms, was indistinguishable from the PTP of strain-matched wild-type mitochondria [21]. Altogether this set of results clearly indicates that the VDAC isoforms are not component of the PTP [22]. Nonetheless, it was recently shown that VDAC can transduce a PTP-sensitizing signal elicited by dephosphorylation of the pro-apoptotic Bcl-2 protein Bad [23].

The PTP is sensitized by the matrix peptidyl-prolyl *cis–trans* isomerase CyP-D. However, the pore is still present in mitochondria and cells obtained from mice where the gene encoding CyP-D was knocked-out. CyP-D ablation increases the Ca^{2+} load required to open the PTP, unmasking inhibition by P_i (see above), and abolishes sensitivity to the CyP-D ligand CsA, conclusively demonstrating that CyP-D is an important regulator of the PTP, but not a component of the channel (reviewed in [10]).

It is plausible that the PTP is composed by proteins spanning the IMM, such as exchangers or ion channels [22]. The PTP is strikingly modulated by ligands of one such protein, the ANT, which exchanges ATP with ADP through the IMM. However, the attractive hypothesis that one or more ANT isoforms are constituents of the pore is not compatible with the finding that mitochondria obtained from ANT1/2$^{-/-}$ double knock-out mice undergo a Ca^{2+}- and oxidant-dependent, CsA-sensitive PT. ANT could be a peripheral PTP regulator, similarly to CyP-D. In fact, ANT null mitochondria require a larger Ca^{2+} load than their wild-type counterparts to open the PTP, which becomes insensitive to ANT ligands [24]. Accordingly, we have found that ANT interacts with CyP-D, and that pore induction triggered by detachment of HK II from mitochondria not only disrupts this interaction, but is inhibited by the ANT ligand bongkrekate [20]. It was claimed that a more recently discovered ANT isoform could be involved in pore formation. However, this is restricted to specific cell types, whereas the PTP is ubiquitously found; in addition, this ANT isoform should be able to promote a CsA-sensitive PT and yet not respond to classical ANT ligands [25].

Recently another IMM transporter, the mitochondrial PiC, has been shown to interact with CyP-D. This interaction is increased in conditions that induce the PTP and reduced by pore desensitizers. Moreover, inhibition of P_i transport desensitizes the PTP in isolated mitochondria [26]. A rigorous genetic analysis is needed to establish whether the PiC is a component of the channel, or a further PTP regulator. Interestingly, as P_i is required for PTP inhibition [7], regulation of PiC by its substrate could affect pore opening [25].

Finally, the disappointing deficiency of information on pore components is further highlighted by the observation that the electrophysiological properties of the hemichannel whose dimers are thought to form the PTP are different from those of all the characterized mitochondrial channels (including VDAC, ANT and PiC), but match those of plasma membrane "maxi-Cl^- channels", whose molecular identity is however undefined [11].

4. PTP and pathogenesis: more and more pore diseases

PTP-dependent mitochondrial dysfunction and PTP deregulation are involved in a variety of diseases characterized by alterations in the molecular mechanisms that control cell death. In pioneering work of the early 1990s, the protective effect of CsA was used to establish a role of the PTP in hepatocytes subjected to oxidative stress or anoxia, and in cardiomyocytes and isolated hearts exposed to ischemia-reperfusion. Later on, the PTP was implicated in many other diseases, including various forms of brain damage, amyotrophic lateral sclerosis, muscular dystrophy caused by collagen VI deficiency, acetaminophen hepatotoxicity and fulminant, death receptor-induced hepatitis (reviewed in [10,27]). In recent years, other disorders have been related to the PTP. In a mouse model of the human lysosomal storage disease GM1-gangliosidosis, GM1-ganglioside induces mitochondrial Ca^{2+} overload, opening of the pore and apoptotic cell death [28]. Dysregulation of mitochondrial Ca^{2+} homeostasis and the following increased sensitivity of the PTP was reported in neuron models of Huntington disease [29,30]. Snake neurotoxins with a phospholipase activity penetrate spinal cord motor neurons and cerebellar granule neurons, bind to mitochondria and facilitate PTP opening [31].

CyP-D knockout mice were used to study several pathologies, particularly of the central nervous system. PTP opening induces brain injury following hypoxic ischemia in the adult animal, whereas in neonatal mice CyP-D protects from ischemic conditions and the pore displays a higher induction threshold [32]. In an amyotrophic lateral sclerosis mouse model, the genetic deletion of CyP-D delays onset of the disease and extends survival [33]. Experimental autoimmune encephalomyelitis mice are a model of multiple sclerosis, as they develop symptoms similar to the human disease when treated with a myelin peptide. Disease progression is dramatically reduced by CyP-D ablation, with a striking inhibition of axonal damage [34]. The amyloid beta (Aβ) peptide, whose massive deposition in the brain is a hallmark of Alzheimer's disease, has been reported to interact with CyP-D. In an Alzheimer's disease mouse model, the absence of CyP-D protects cortical neurons from PT and cell death induced by Aβ and Ca^{2+}, improves learning and memory and synaptic function [35] also in aged animals [36]. It should also be mentioned that CyP-D knockout mice have an interesting psychological phenotype with increased anxiety, facilitation of avoidance behavior; and that they develop adult-onset obesity independent of food and water intake [37]. Finally, CyP-D ablation protects from cell death in ischemic renal injury [38].

PTP opening has been examined extensively in cardiac ischemia/reperfusion injury. Ischemia is defined as an imbalance between oxygen supply and demand. In these conditions, mitochondria halt ATP synthesis, cells accumulate glycolytic lactate, and in the attempt to restore intracellular pH activate the plasma membrane Na^+/H^+ exchanger, whereas the Na^+/K^+-ATPase activity is suppressed. The ensuing increase in intracellular Na^+ stimulates the Na^+/Ca^{2+} exchanger, and together with the block of reticular Ca^{2+}-ATPases this increases intracellular Ca^{2+}. Moreover, the ischemic cell can maintain an oxygen level sufficient to generate ROS, and a burst of ROS, with further progression of Ca^{2+} overload caused by the Na^+–Ca^{2+} exchanger, is generated in the first minutes of reperfusion. Altogether, Ca^{2+} overload and even more importantly oxidative stress [39] provide ideal circumstances for opening of the PTP, leading to cytochrome *c* release and cell death [40]. A protective effect of PTP desensitization through CsA

has been demonstrated in different models of ischemia followed by reperfusion. At present, inhibition of pore opening by non-immunosuppressive analogs of CsA or antioxidants is the most promising therapeutic approach [22,25,41].

Recent data suggest that PTP dysregulation is involved in tumorigenesis. Neoplastic cells are more resistant than non-transformed cells to PTP-induced mitochondrial damage, and this is involved in resistance to chemotherapeutics and in the ability acquired by tumor cells to escape apoptosis induced by a variety of stressful conditions, such as hypoxia or detachment from the extracellular matrix [10]. PTP opening was recently found to trigger death of keratinocytes exposed to UV light, whereas CsA-induced PTP desensitization induced tumor formation [42]. In the rodent model of hepatocarcinogenesis caused by 2-acetylaminofluorene, desensitization of the PTP is an early adaptive response of hepatocytes that acts as a tumor-promoting event [43]. Also other conditions that predispose to liver cancer (alcoholic liver disease, chronic hepatitis C and cholestasis) were proposed to involve PTP deregulation [10]. In this framework, we have recently found that transformation of a prostate cell model induces PTP desensitization and resistance to cell death [44].

5. Switching a pore: signal transduction to the PTP

In the past, extensive analyses have dissected the factors that regulate the PTP in situ [6]. Nonetheless, extreme caution must be exerted when these data are extrapolated to PTP modulation in the living cell. The continuous crosstalk between mitochondria and other cell compartments suggests that several proteins can play regulatory roles on the pore, connecting signaling cascades known to control the apoptotic process with the PTP, as this is a key switch in the cell commitment to death.

An example of these regulatory components is hexokinase (HK). HK initiates all major pathways of intracellular glucose utilization, and type II hexokinase (HK II) is highly expressed in most cancer cells, where it mainly localizes on the OMM (Fig. 1A).

Mitochondrial HK II is extremely important in neoplastic transformation. In highly glycolytic, aggressive tumors, it fosters cell growth in the hypoxic conditions of primary tumor mass accrual. This would contribute to the Warburg effect, i.e. to the uncoupling between glycolysis enhancement and oxygen availability, supporting cell proliferation when the neoplastic mass outgrows the surrounding blood vessels. Accordingly, in hepatocellular carcinoma cells hypoxia stimulates growth via HK II induction, whereas HK II inhibition induces apoptosis [45], and release of HK II from mitochondria prompts apoptosis in glioma cells [46]. Detachment of HK II from mitochondria by a selective peptide induces PTP opening and cell death in tumor cell models and in cardiomyocytes [20,47]. In experiments with CsA and CsA derivatives, and with cells and mitochondria derived from CyP-D knock-out mice, we have shown that CyP-D enhances PTP opening and the ensuing apoptosis triggered by detachment of HK II from mitochondria [20]. These observations suggest that mitochondrial HK II delivers a survival signal that stabilizes the PTP in the closed conformation, whereas HK II detachment from mitochondria would propagate a conformational change to molecules of the inner mitochondrial membrane, eventually leading to pore opening (Fig. 1B). Moreover, in this model the knockout of CyP-D inhibits apoptosis, at variance from what has been reported in other experimental settings [48,49]. However, it remains unclear what mediates the interaction between HK II, which is located on the mitochondrial surface, and PTP regulators or components in the inner mitochondrial compartments.

Signal transduction pathways converge on mitochondrial HK II (Fig. 1A and B). This is of pivotal importance in cancer, because tumor cells are endowed with hyper-activation of anabolic pathways. Thus, by integrating information from growth factor signalling, mitochondrial HK II could act as a potent anti-apoptotic "porekeeper" to suppress cell death either in conditions of adequate nutrition or when kinase pathways are aberrantly boosted by malignant transformation. The survival kinase Akt, which is activated by growth factors and in most tumor cells, promotes HK II binding to mitochondria. Indeed, Akt phosphorylates both HK II itself or glycogen synthase kinase 3β (GSK3β). Akt-dependent phosphorylation of mitochondrial HK II inhibits Ca^{2+}-induced cytochrome *c* release [50], and association of HK II to the OMM is favoured when GSK3β is inactivated by Akt phosphorylation [51,52]. Accordingly, activation of GSK3β was shown to induce release of HK II, enhancing susceptibility to cell death [51,52]. This was proposed to require GSK3β phosphorylation of VDAC, which would displace Bcl-2 from its interaction with VDAC, favoring the binding between VDAC and the pro-apoptotic Bax/Bak proteins in conditions of growth factor deprivation [51,52]. This would result in increased sensitivity of mitochondria to PTP induction. However, mitochondrial displacement of HK II induced cell death also after insulin-dependent Akt activation or in cells lacking VDAC1 and 3 (and without any detectable binding between HK II and residual VDAC2) [20], and even in the absence of Bax and Bak [53] (also discussed in [8]).

PTP regulation by kinase signalling has been widely investigated, particularly in cardiac models. A group of kinases, termed RISK (reperfusion injury salvage kinase), confers cardioprotection when activated during post-ischemic myocardial reperfusion or ischemic pre- and post-conditioning [54]. Several agents, including growth factors and hormones, reduce myocardial infarct size by activation of the RISK pathway (Fig. 2). RISKs include the survival kinases Akt and Erk1/2, protein kinase Cε (PKCε), protein kinase G (PKG) and p70s6K, and protect from cell death by phosphorylation of Bcl-2 family proteins and inhibition of the mitochondrial PTP, with a possible interplay between these events [54].

A substrate of the RISK pathway is GSK3β which, unlike most kinases, is constitutively active in unstimulated cells, exerting a tonic inhibitory effect on its downstream targets [55]. GSK3β is inactivated by serine phosphorylation induced by a variety of signalling pathways, whereas autophosphorylation at a specific tyrosine increases its overall catalytic efficiency. Deregulation of this GSK3β fine tuning has been associated with a variety of diseases, such as neurodegenerative diseases, cancer and ischemia/reperfusion injury, which involve disturbances in apoptosis regulation [55]. In cardiomyocytes, receptor tyrosine kinase or G protein-coupled receptor activation, or ROS production induced by reoxygenation after prolonged hypoxia lead to activation of diverse RISK components, and ultimately converge on PTP desensitization downstream of inactivating GSK3β phosphorylation [56]. Accordingly, expression of a constitutively active GSK-3β failed to protect myocytes against oxidative stress, whereas transfection with a kinase-dead enzyme was protective [47,57,58]. Moreover, infarct size in mice was reduced by CsA and by a GSK3β inhibitor, whereas in mice expressing a constitutive active GSK3β only CsA was effective [59]. As in mitochondria from wild-type animals the PTP inhibitory effect of CsA and of the GSK3β inhibitor were additive, it was proposed that GSK3β facilitates pore opening independently of CyP-D [59].

This PTP regulation is operated by changing the ROS and/or Ca^{2+} threshold of PTP opening, and should be elicited by a mitochondrial pool of GSK3β acting as an integration point to funnel a multiplicity of survival pathways to target(s) at or in close proximity to the PTP (Fig. 2). Nonetheless, further layers of complexity are involved in this picture. For instance, a knock-in mouse with a constitutive active GSK3 (i.e. that cannot be phosphorylated on regulatory serine) displayed heart protection when pre- or post-

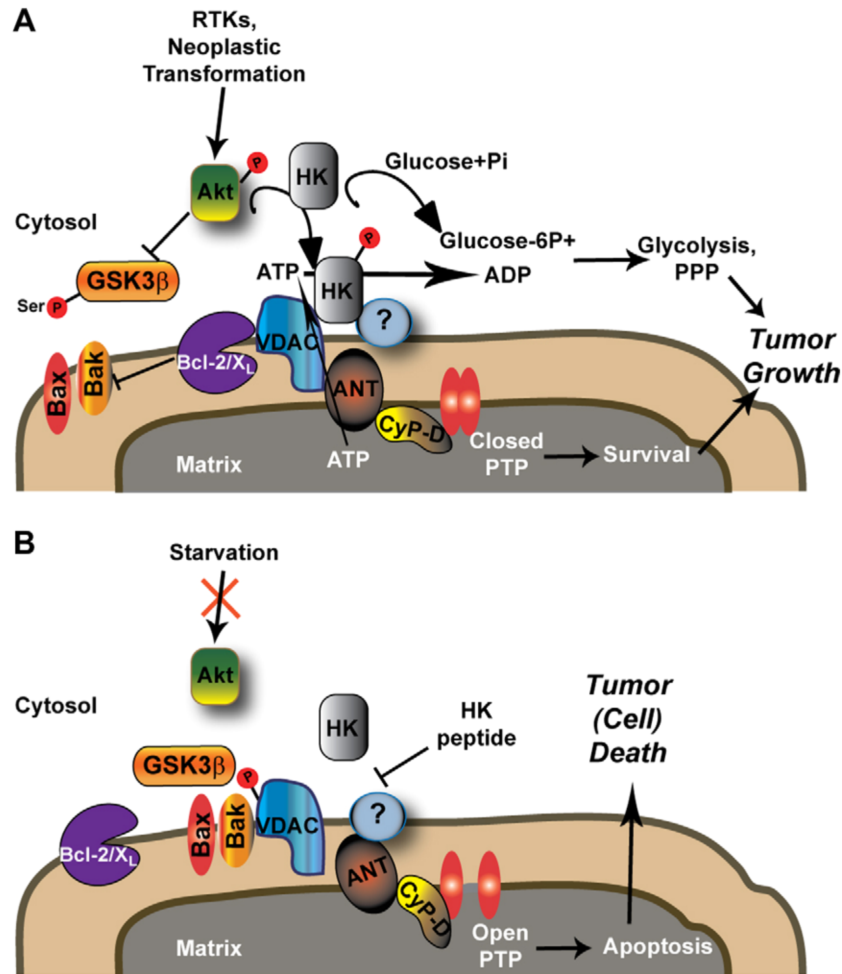

Fig. 1. PTP regulation by mitochondrial hexokinase (HK). (A) Akt activation by receptor tyrosine kinases (RTKs) or during neoplastic transformation keeps HK bound to the mitochondrial surface, both through a direct HK phosphorylation and through an inactivating serine phosphorylation of GSK3β. Inactive GSK3β cannot phosphorylate VDAC, favoring its binding to HK and to anti-apoptotic Bcl-2 family proteins, and displacing the binding of pro-apoptotic Bax/Bak. However, VDAC is dispensable for HK interaction with mitochondria, suggesting that (an) unknown partner(s) is/are involved in this process. HK utilizes the ATP synthesized by mitochondria to start glucose metabolism, and stabilizes the PTP in a closed state. (B) Either Akt inactivation or treatment with a HK peptide induce HK detachment from the outer mitochondrial membrane, possibly leading to conformational changes of ANT and CyP-D and eventually to PTP opening and cell death. GSK3β is activated by Akt inhibition. The ensuing VDAC phosphorylation leads to Bcl-2/X_L displacement and favors Bax/Bak activation, but PTP opening induced by HK detachment from mitochondria does not require VDAC.

conditioning protocols were applied, and inhibitors of GSK3β failed to limit infarct size in these animals [60]. Hence, these knock-in mice might have developed alternative ways to negatively regulate GSK3β (discussed in [47]). In accord with this possibility, it must be stressed that GSK3β has further regulatory mechanisms, including phosphorylation at additional Ser residues, complex formation with scaffold proteins, priming of substrates and of the enzyme itself and intracellular translocation (reviewed in [41,47,55]). Moreover, GSK3β could indirectly regulate the PTP in several ways. These include the already discussed modulation of HK II binding to mitochondria, regulation of the interaction between ANT and CyP-D, mitochondrial translocation of pro-apoptotic Bcl-2 family members, degradation of anti-apoptotic Bcl-2-like proteins [41,47]. GSK3β inhibition also reduces infarct size via the opening of ATP-dependent K^+ channels and the ensuing PTP closure [61], and phosphorylation of mitochondrial serine/threonine protein phosphatases (e.g. the subunit B of protein phosphatase 2A that possesses a consensus site for GSK3β) could directly affect the pore. Indeed, a mitochondrial matrix-targeted protein phosphatase 2C family member (PP2Cm) was shown to inhibit PTP opening and to be essential for cell survival [62], whereas PTP-mediated apoptosis was shown to be inducible by the protein phosphatases 2B [63] and SH2-containing tyrosine phosphatase 1 [64] in different cell models.

It is possible that other kinase/phosphatase signaling pathways can converge on the PTP through GSK3β regulation, or alternatively bypass it to directly impinge on the pore. PKCε plays a critical role in cardioprotective signalling pathways, targeting mitochondrial ATP-sensitive K^+ channels, respiratory chain components, ROS production and both ANT and VDAC. PKCε can inhibit PTP opening in a phosphorylation-dependent reaction and also in isolated mitochondria, conferring protection from reperfusion-induced cell death [65,66]. The master survival kinase Akt protects the ischemic heart through inhibition of the PTP [67], and growth factors provided before ischemia reduce infarct size and increase the fraction of activated Akt in mitochondria, where it complexes with ANT upon reperfusion [68]. As in the case of GSK3β, Akt could also indirectly modulate pore opening through the targeting of Bcl-2 family proteins, either affecting their subcellular localization or their degradation or regulating them at the transcriptional level [69]. In hepatoma cells Akt inactivation by oxidative stress induces mitochondrial injury associated with opening of the PTP but independently of both GSK3β and Bcl-2 family proteins [70]. In pheochromocytoma cells, GSK3β causes mitochondrial pore open-

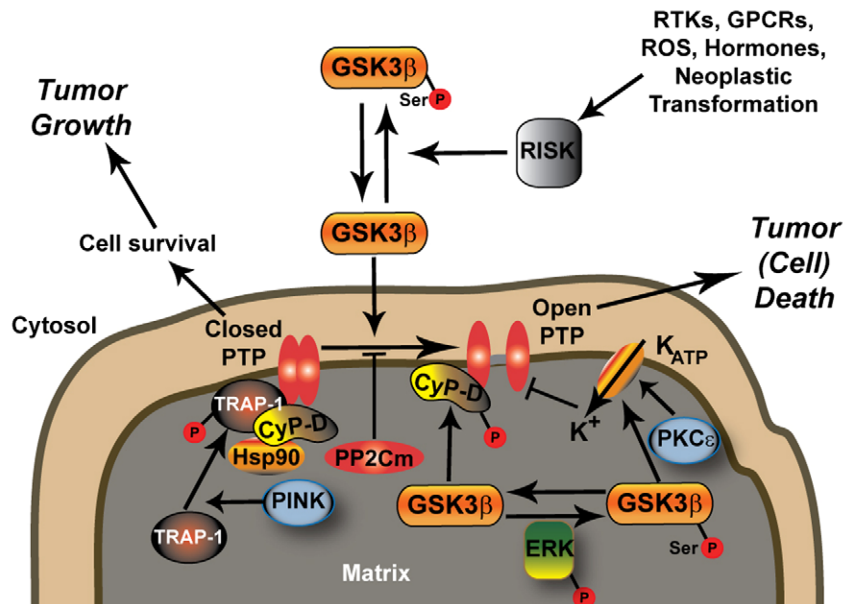

Fig. 2. Kinase signalling to the PTP. Several kinase pathways converge on PTP regulation. A common target, at least during post-ischemic reperfusion, is GSK3β, which is inactivated by a group of survival kinases termed RISK (reperfusion injury salvage kinases). A small pool of GSK3β is in the mitochondrial matrix, where it favors PTP opening, possibly through CyP-D phosphorylation. In tumors, mitochondrial GSK3β is inactivated by ERK-dependent phosphorylation, raising the threshold for pore opening and therefore enhancing cell survival. GSK3β inactivation, and PKCε activity, could also contribute in keeping closed the PTP by opening mitochondrial K_{ATP} channels. CyP-D regulation of the pore is also influenced by the chaperones Hsp90 and TRAP-1; TRAP-1 is activated by the Ser/Thr kinase PINK1. Finally, PTP opening is inhibited by the phosphatase PP2Cm through unknown mechanisms. RTKs: receptor tyrosine kinases; GPCRs: G protein coupled receptors.

ing and the subsequent cell death via upregulation of the stress kinase c-Jun N-terminal kinase [71].

Several factors render extremely difficult a better definition of the effects of kinase/phosphatase signalling on the PTP. As it is always the case for (de)phosphorylation events, critical components are the timing, duration and intensity of the signal, which are in turn dependent on the cell type, the subcellular localization of scaffolding, adapter and regulatory proteins, on the assembly of multimeric complexes and even on specific non-proteinaceous components (e.g. lipid species). In addition, the absence of recognized mitochondrial import sequences in most known kinases ask for laborious investigations to assess the direct interaction between these enzymes and molecules located in inner mitochondrial compartments. The limited knowledge of proteins that regulate the pore, and the lack of information on PTP components, make an arduous task the subtle dissection of the interplay between kinase pathway and the pore, and of its functional meaning. In this framework, an important role can be played by molecular chaperones (Fig. 2). A chaperone homologous to heat shock protein 90 (Hsp90), termed tumor necrosis factor receptor-associated protein-1 (TRAP-1), is localized in mitochondria of tumor cells and of nervous tissue, but not in most normal tissues [72,73]. Both TRAP-1 and the mitochondrial fraction of Hsp90 interact with CyP-D and antagonize its function of PTP sensitization [72]. Short hairpin RNA-mediated knockdown of TRAP-1 enhances a CsA-inhibitable cell death, whereas TRAP-1 overexpression confers resistance to pro-apoptotic drugs [72], suggesting that TRAP-1/Hsp90 are novel mitochondrial survival factor acting as PTP inhibitors, and constitute possible targets of mitochondria-directed chemotherapeutics drugs [74]. Importantly, TRAP-1 was shown to be substrate of the Ser/Thr kinase PTEN induced kinase 1 (PINK1) in the mitochondrial matrix, and this phosphorylation was required to prevent oxidative-stress-induced apoptosis [73]. Overexpression of PINK1 blocked PTP-dependent cell death [75], whereas PINK1 deficiency lowers the threshold of PTP opening by impairing intracellular Ca^{2+} homeostasis and enhancing ROS production in neurons [76].

Altogether, these results further indicate that phosphorylation events can be crucial in the regulation of the PTP, and that molecular chaperones as TRAP-1 might be a link between kinase pathways and the pore. Furthermore, this could have important pathogenic implications, both in tumors, where TRAP-1 overexpression could substantially contribute to “lock” the PTP in front of diverse insults, and in neurodegenerative diseases. In fact, it was shown that the cell survival function of PINK1 requires its ability to phosphorylate TRAP-1, and that this activity gets lost in the presence of PINK1 mutations causing an autosomal recessive form of Parkinson disease [73,77].

We have recently shown that also CyP-D is phosphorylated on serine/threonine residues in tumor cell models [44]. A stepwise protease digestion assay indicated that a small portion of both extracellular signal regulated kinase (ERK) and GSK3 was located in the mitochondrial matrix in several neoplastic cell models, and that both kinases directly interacted with CyP-D. Mitochondrial ERK turned out to be constitutively active after v-Ki-Ras dependent transformation or in diverse tumor models, conferring resistance to death stimuli acting as PTP inducers. This protection could be ablated by inhibiting ERK with the drug PD98059 or with a selective ERK activation inhibitor peptide. Notably, these treatments enhanced GSK-3-dependent phosphorylation of CyP-D and PTP opening, and the effect on the pore was increased after CyP-D overexpression and absent in CyP-D knock-out cells. Accordingly, a recombinant GSK3 could phosphorylate CyP-D in vitro, and an in silico analysis identified possible GSK-3 target residues on CyP-D. Conversely, ERK activation prompted the inhibitory phosphorylation of GSK3 and abolished CyP-D phosphorylation, and GSK-3 pharmacological inhibition protected from PTP opening.

Thus, in diverse tumor cell models resilience to undergo cell death is caused by ERK activity, which could directly impinge upon pore opening through the negative regulation of CyP-D phosphorylation by inhibiting the mitochondrial pool of GSK3. Mitochondria-specific ERK activation might provide a key advantage during neoplastic transformation, by placing the death/survival

mitochondrial rheostat in an anti-apoptotic mode. To make this picture more complex, it must be highlighted that ERK is a GSK-3 priming kinase, requiring the activity of a second kinase for complete GSK-3 inhibition; and that GSK-3 itself needs a priming kinase to phosphorylate its targets. Moreover, these reactions must be finely tuned by the action of phosphatases.

From the above, we postulate that a network of phosphorylation events might control PTP regulation by modulating the activity of molecular chaperones such as TRAP-1 or CyP-D. Dysregulation of kinase signalling pathways is a hallmark of a number of diseases, in primis cancer, and the mitochondrial pore could constitute an important downstream effector in conferring crucial pathogenic traits, such as alterations in cell death responses, and an attractive pharmacological target.

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