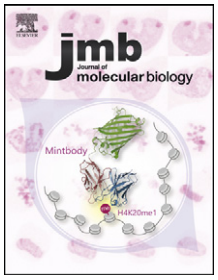

A Novel *In Vitro* CypD-Mediated p53 Aggregation Assay Suggests a Model for Mitochondrial Permeability Transition by Chaperone Systems

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Abstract

Tissue necrosis as a consequence of ischemia–reperfusion injury and oxidative damage is a leading cause of permanent disability and death worldwide. The complete mechanism by which cells undergo necrosis upon oxidative stress is not understood. In response to an oxidative insult, wild-type p53 has been implicated as a central regulatory component of the mitochondrial permeability transition (mPT), triggering necrosis. This process is associated with cellular stabilization and translocation of p53 into the mitochondrial matrix. Here, we probe the mechanism by which p53 activates the key mPT regulator cyclophilin D (CypD). We explore the involvement of Trap1, an Hsp90-related mitochondrial matrix protein and a member of the mitochondrial unfolded protein response, and its ability to suppress mPT in a p53-dependent manner. Our study finds that catalytically active CypD causes strong aggregation of wild-type p53 protein (both full-length and isolated DNA-binding domain) into amyloid-type fibrils *in vitro*. The responsible CypD residues for this activity were mapped by NMR to the active site amino acids R55, F60, F113, and W121. The data also present a new proline isomerization assay for CypD by monitoring the aggregation of p53 as an indicator of CypD activity. Moreover, we find that the inhibition of Trap1 by the mitochondria-specific HSP90 ATPase antagonist Gamitrinib strongly sensitizes primary mouse embryonic fibroblasts to mPT and permeability transition pore opening in a p53- and CypD-dependent manner. We propose a mechanism by which the influx of unfolded p53 into the mitochondrial matrix in response to oxidative stress indirectly activates the normally inhibited CypD by displacing it from Trap1 complexes. This activates CypD's isomerase activity. Liberated CypD then isomerizes multiple proteins including p53 (causing p53 aggregation) and the structural components of the mPTP pore, inducing pore opening. This working model can now be tested in the future.

Introduction

Ischemia–reperfusion (IR) injury can cause irreversible tissue damage and necrosis in vital organs such as the brain (ischemic or hemorrhagic stroke) and heart (myocardial infarction), and reperfusion injury is a leading cause of permanent disability and death worldwide [1]. IR injury leads to increased intracellular concentrations of reactive oxygen species (ROS) and increased cytosolic Ca^{2+} influx into the mitochondrial matrix [2]. Together, these events lead to dissipation and collapse of the actively maintained electrochemical proton gradient ($\Delta\Psi m$) across the inner mitochondrial membrane due to the sudden opening of the mitochondrial permeability transition pore (mPTP) in the inner mitochondrial membrane. The mPTP is an elusive non-selective pore for small solutes and water that is impermeable under normal physiologic conditions but opens abruptly in response to prolonged oxidative stress, an event called mitochondrial permeability transition (mPT) [2,3]. This causes ion influx that dissipates $\Delta\Psi m$ and shuts down oxidative phosphorylation and ATP production, ending in catastrophic energetic failure [3]. Concomitantly, water influx causes matrix swelling, rupture of the rigid outer mitochondrial

membrane, and release of all sequestered cell death factors from the intermembraneous space. Recent studies identified several key players involved in mPT. They include the central regulatory protein cyclophilin D [CypD; gene name PPIF, UniProt ID: P30405, Protein Data Bank (PDB) ID: 2Z6W, not to be confused with the larger 40 kDa nuclear and cytosolic CypD otherwise known as CyP40 (Supplementary Fig. 1a)], an exclusively mitochondrial matrix prolyl isomerase whose enzymatic activity is essential to trigger mPT [4–6], and the stress sensor p53 (TP53) under conditions of oxidative damage [7–12]. Structural pore components encompass the c-subunit ring [13,14], the oligomycin-sensitivity conferring protein (OSCP) [15] of the F_1F_o-ATP synthase, and, more recently, the product of the Spastic Paraplegia 7 gene [16]. Despite these advances, the full assembly of the mPTP is not understood, and more importantly, the mechanism by which the pore opening is triggered remains unknown.

p53 is an extensively studied nuclear transcription factor that acts as a potent tumor suppressor by rapidly and broadly responding to DNA damage signals *via* activating genes involved in cell cycle arrest or senescence, DNA repair, and apoptosis as effective anticancer mechanisms. Moreover, wild-type (WT) p53 protein has additional pro-death functions directly at the mitochondria, including driving transcription-independent apoptosis [17].

Recent work has shown that a fraction of stress-induced p53 protein translocates to mitochondria to interact with multiple members of the Bcl2 family and directly drive apoptosis through permeabilization of the outer mitochondrial membrane [18]. According to a long-standing paradigm, p53 controls apoptosis but plays no role in necrosis. However, in response to oxidative stress, we recently established a necrotic mitochondrial p53 program [7]. We showed that upon hypoxia and oxidative stress, cytoplasmic p53 translocates into the mitochondrial matrix and participates in necrosis by triggering mPT through interaction with CypD [7]. While the apoptotic p53 cell death program requires Bax and Bak, the necrotic p53 cell death program requires CypD: purified p53 protein opens the mitochondrial permeability transition pore (PTP) pore in isolated mitochondria independent of Bax and Bak but dependent on CypD [7]. Conversely, $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) are protected from oxidative-stress-induced mPT and mPTP opening. Direct targeting of p53 to mitochondrial matrix induces mPT and necrosis in a CypD-dependent manner, and oxidative-stress-induced PTP opening and necrosis are largely transcription independent. Intriguingly, a robust p53–CypD complex forms during mouse brain IR injury (stroke model). In contrast, reduction of p53 levels or cyclosporine A (CsA) pretreatment of mice prevents this complex and correlates with effective stroke protection [7]. However, details of the p53•CypD interaction and the mechanism by which p53 triggers the opening of the mPTP have yet to be investigated.

The major event during IR injury leading to sudden collapse of mitochondrial function and energy catastrophe is the formation and prolonged opening of the mPTP, following the insult by ROS and/or Ca^{2+}. While it has been shown that the mPTP can be transiently opened and closed to serve as a physiologic Ca^{2+} release channel [19], it is the prolonged opening of the pore that is required for necrotic cell death [20]. The exact proteins that make up the mPTP have evaded researchers for over two decades, and the only genetically proven and consistently indispensable component has been the obligatory regulatory factor CypD [4–6]. Genetic deletion of CypD [21–23] or inhibition of CypD with the highly specific pan-cyclophilin inhibitor CsA [7,24] prevents mPTP opening and mPT *in vivo*. It is thought that the structural pore component of the mPTP itself is composed of the c-subunit ring of the F_1F_o-ATP synthase [14] and that CypD binds distally to the OSCP subunit of the F_1F_o-ATP synthase [13]. Binding of CypD to OSCP is hypothesized to increase the PTP's apparent affinity for Ca^{2+}, in turn sensitizing the pore to opening [20].

Over 1500 different proteins can be found in mammalian mitochondria [25]. However, the mitochondrial genome only harbors 13 protein-coding genes [26], indicating that the vast majority of mitochondrial proteins including p53 and CypD are encoded in the nucleus and imported from the cytosol. As such, mitochondria heavily depend on chaperone proteins to maintain proper protein-folding homeostasis [27]. These mitochondrial chaperones are also regulators of mPT, contributing to a cytoprotective chaperone network that antagonizes CypD-dependent cell death [28–32]. Conversely, and consistent with this vital role, mitochondrial chaperones are involved in the mitochondrial unfolded protein response (mtUPR) [33] that is triggered by a variety of mitochondrial stressors including ROS [34]. Notably, both CypD and p53 interact with members of the mtUPR including mitochondrial mtHSP60 [28,35], mtHSP90 [36], and the mitochondrial heat-shock protein (HSP) 90 homolog Trap1 [29]. These interactions beckon the question of whether or not mitochondrial chaperones may also be involved in p53-mediated mPT.

To better understand the mechanism of how p53 is involved in oxidative-stress-induced opening of the mPTP, we employed complementary biophysical methods here to explore the p53•CypD interaction. We find that CypD and p53 engage in a transient enzyme–substrate interaction and not in a stable protein–protein complex. Rather, the isomerase activity of CypD leads to the aggregation of its substrate, p53. NMR and biochemical methods were used to identify catalytic CypD residues directly involved in aggregation of p53. We monitor CypD-induced p53 aggregation in a continuous spectrophotometric assay