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Hsp60 REGULATION OF THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE IN TUMOR CELLS

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Abstract

Mitochondrial apoptosis plays a critical role in tumor maintenance and dictates the response to therapy, *in vivo*, but the regulators of this process are still largely elusive. Here, we show that the molecular chaperone Heat Shock Protein-60 (Hsp60) directly associates with cyclophilin D (CypD), a component of the mitochondrial permeability transition pore. This interaction occurs in a multi-chaperone complex comprising Hsp60, Hsp90 and Tumor Necrosis Factor Receptor-Associated Protein-1 (TRAP-1), selectively assembled in tumor, but not normal mitochondria. Genetic targeting of Hsp60 by small interfering RNA (siRNA) triggers CypD-dependent mitochondrial permeability transition, caspase-dependent apoptosis and suppression of intracranial glioblastoma growth, *in vivo*. Therefore, Hsp60 is a novel regulator of mitochondrial permeability transition, contributing to a cytoprotective chaperone network that antagonizes CypD-dependent cell death in tumors.

Keywords

Hsp60; chaperone; cyclophilin D; mitochondria; apoptosis; permeability transition

INTRODUCTION

Disparate cell death stimuli initiate mitochondrial apoptosis (1) via a stepwise cascade that includes dissipation of the organelle inner membrane potential, swelling of the matrix, rupture of the outer membrane, and release of apoptogenic proteins in the cytosol, most notably cytochrome c (2). This process of mitochondrial permeability transition (3) is commonly subverted in tumors, resulting in enhanced cell viability, adaptation and resistance to anticancer therapy (4). Although anti-apoptotic Bcl-2 proteins oppose mitochondrial outer membrane permeability (5), and are often exploited in cancer, the existence of additional, “mitochondrial-intrinsic” pathways that counter apoptosis selectively in tumors, *in vivo* has remained unclear.

Potential mediators of such mechanism(s) may include molecular chaperones of the Heat Shock Protein (Hsp) family, including Hsp90, and its related molecule, TRAP-1, which are abundantly expressed in organelles of tumor, but not most normal cells, *in vivo* (6). These mitochondrial Hsp90s associate with the matrix immunophilin, cyclophilin D (CypD) (6), a

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DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

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pro-apoptotic component of a mitochondrial permeability transition pore (7,8), and antagonize its function via protein (re)folding, thus preserving organelle integrity and shutting off the initiation of cell death (9).

In this study, we asked whether a broader, mitochondrial chaperone network is exploited to quench CypD activity selectively in tumors. We found that Hsp60 (10), a chaperone with critical roles in mitochondrial protein folding (11), and over-expressed in tumors (12), is a novel cytoprotective regulator of CypD, and this pathway is required for tumor growth, in vivo.

MATERIALS AND METHODS

Cells and cell cultures

Human embryonic kidney HEK293T, normal human fibroblasts (NHF), breast adenocarcinoma MCF-7, colorectal adenocarcinoma HCT116, and glioblastoma U87 and LN229 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). CypD-directed short hairpin RNA (Open Biosystems cat. n. RHS3979-9616542, RHS3979-9616543) in pLKO.1 lentivirus vector was co-transfected (0.5 µg) in HEK293T cells with 0.33 µg pCMVdel8.2 and 0.2 µg pMDG (VSVG envelope glycoprotein) using Lipofectamine 2000 (Invitrogen). Supernatants were harvested after 48 h, filtered through a 0.45 µm pore size filter, and used to infect LN229 cells in the presence of 4 µg/ml of polybrene. Cells were selected in 2 µg/ml puromycin for 7 d. Alternatively, U87 cells were infected with pGIPZ lentiviral vector encoding control shRNA (RHS4349, Open Biosystems) or Hsp60-directed shRNA (RHS4531 clone ID#V2LHS-191368, Open Biosystems), with selection of stable clones in puromycin-containing medium.

Size exclusion liquid chromatography

Mitochondrial extracts from normal mouse liver or HCT116 cells were prepared as described (6). Lysates were fractionated by fast protein liquid chromatography (FPLC) on a Superdex 200 10/30 column (Amersham Biosciences) with elution at 4°C in 50 mM Tris, 100 mM NaCl and 0.5% IGEPAL (Sigma-Aldrich), pH 7.0, at 0.4 ml/min, and collection of 0.5- or 1-ml fractions. The columns were calibrated with protein standards (GE Healthcare Biosciences), blue dextran (2,000 kDa), ferritin (440 kDa), aldolase (158 kDa), alcohol dehydrogenase (150 kDa), conalbumin (75 kDa), and ovalbumin (43 kDa).

Protein-protein interactions and transfection

Expression of recombinant proteins in BL21 *E.Coli* strain, pull down, immunoprecipitation or Western blotting was as described (6,12). Gene silencing by small interfering RNA (siRNA) has been described (6,12).

Mitochondrial dysfunction, apoptosis and cell proliferation

Mitochondrial membrane potential was quantified by JC-1 staining and flow cytometry (12). Caspase-dependent apoptosis was determined by DEVDase activity and propidium iodide (PI) staining and multiparametric flow cytometry (6). Genetically engineered cultures (5×10^4) were analyzed for cell proliferation or viability by direct cell counting, or MTT (9).

In vivo glioblastoma model

All experiments involving animals were approved by an Institutional Animal Care and Use Committee. U87 cells expressing luciferase (U87-Luc) and stably transduced with scrambled shRNA or Hsp60-directed shRNA (5 animals/group) were stereotactically implanted (1×10^5) in the right cerebral striatum of immunocompromised nude mice (Charles

River Laboratories). Tumor growth was quantified weekly by bioluminescence imaging using a Xenogen In Vivo Imaging System after i.p injection of 110 mg/kg D-luciferin. At the end of the experiment, tumor samples from the two animal groups were harvested, and analyzed by immunohistochemistry, as described (6).

Statistical analysis

Data were analyzed by two-sided unpaired t-tests using a GraphPad software package (Prism 4.0) for Windows. Animal survival was quantified by a Kaplan-Meier curve. A *p* value of 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

We began this study by fractionating mitochondrial extracts of tumor cells (HCT116) or normal mouse liver to identify novel regulators of CypD (7,8)-dependent permeability transition (2). Size-exclusion FPLC produced comparable elution profiles from the two mitochondrial sources (Fig. 1A), and similar protein yield (Fig 1B). Partially overlapping mitochondrial fractions eluted from HCT116 extracts contained Hsp90, TRAP-1 (6), and Hsp60 (12) (Fig. 1C). A pool of CypD co-eluted in the same fractions containing Hsp60 and TRAP-1, and, to a lesser extent, Hsp90 (Fig. 1C). A second pool of CypD eluted in later fractions, potentially corresponding to free, i.e. uncomplexed protein (Fig. 1C). As control, CypD-reactive fractions contained the adenine nucleotide translocator (ANT), which associates with CypD in a permeability transition pore (2), whereas a cytoplasmic marker, Ran, was negative (Fig. 1C). In contrast, FPLC fractions eluted from normal liver mitochondria contained low levels of Hsp90 and Hsp60, and barely detectable TRAP-1 (Fig. 1D), consistent with the low expression of these chaperones in normal mitochondria (6,12). In addition, CypD did not co-elute with any of the mitochondrial chaperones in normal liver extracts, and emerged only as free, uncomplexed protein (Fig. 1D).

In capture assays with HCT116 mitochondrial extracts, endogenous CypD bound recombinant Hsp90 (6), as well as Hsp60, whereas GST was unreactive (Fig. 2A). Bacterially expressed Hsp60, but not GST, bound recombinant CypD, and TRAP-1 (6) in the absence of cellular proteins, indicating that these interactions were direct (Fig. 2B). When analyzed *in vivo*, immune complexes precipitated with an antibody to Hsp60 from tumor mitochondria contained endogenous CypD, whereas non-binding IgG was negative (Fig. 2C). Although Hsp60 (and TRAP-1) also associated with survivin (12), *in vivo* (Supplementary Fig. S1A), and *in vitro* (Supplementary Fig. S1B), mitochondrial survivin did not directly bind recombinant CypD in a pull down assay (Supplementary Fig. S1B). Consistent with the assembly of an Hsp60-CypD complex selectively in tumor cells, recombinant Hsp60 did not bind CypD in NHF (Fig. 2D, *left*). Conversely, both normal and tumor human cell lines expressed comparable levels of CypD (Fig. 2D, *right*).

siRNA silencing of Hsp60 (Supplementary Fig. S2A) triggered mitochondrial permeability transition in tumor cells, with loss of membrane potential (Supplementary Fig. S2B), cytochrome c release (Fig. 3A), and caspase-dependent cell death (Fig. 3B) (12). Conversely, inhibition of CypD peptidyl-prolyl *cis,trans* isomerase activity with cyclosporine (CsA) (6) reversed mitochondrial depolarization (Supplementary Fig. S2C), cytochrome c release (Fig. 3A), and apoptosis (Fig. 3B) induced by Hsp60 silencing. Similar results were independently obtained with siRNA knockdown of CypD, which rescued cytochrome c release (Supplementary Fig. S2A) and loss of mitochondrial membrane potential (Supplementary Fig. S2B) associated with Hsp60 depletion. Further, LN229 clones with stable shRNA knockdown of CypD (Fig. 3C) also exhibited reduced mitochondrial depolarization (Supplementary Fig. S3A) and cytochrome c release (Supplementary Fig. S3B) after Hsp60 targeting, compared to scrambled transfectants. Consistent with a role of

CypD as target of Hsp60 cytoprotection, Hsp60 knockdown reduced the viability of LN229 cells transduced with non-targeting shRNA, but had no effect on cells with stable shRNA silencing of CypD (Fig. 3D).

To test the relevance of Hsp60 regulation of CypD for tumor cell survival, *in vivo*, we next infected U87-Luc glioblastoma cells with lentivirus encoding scrambled shRNA or Hsp60-directed shRNA. Stable shRNA knockdown of Hsp60 (Fig. 4A, *left*) significantly inhibited U87 cell proliferation in the presence of serum (10%), compared to control cultures (Fig. 4B, *right*). Stereotactic injection of control U87-Luc cells in the right cerebral striatum of immunocompromised mice gave rise to exponentially growing intracranial glioblastomas (Fig. 4B, *top*), quantified by bioluminescence imaging (Fig. 4B, *bottom*), with death of all animals within 25 d (Fig. 4C). In contrast, stable silencing of Hsp60 in U87-Luc cells suppressed the growth of intracranial glioblastoma, *in vivo* (Fig. 4B), and significantly prolonged survival of all animals in this group (Fig. 4C). Tumors in the Hsp60 shRNA-targeted group retained nearly complete loss of Hsp60 protein expression at the end of the experiment, compared to scrambled shRNA, by immunohistochemistry (Fig. 4D).

In summary, we identified Hsp60 as a novel regulator of CypD-mediated mitochondrial permeability transition (2), selectively in tumors (1). These findings point to a broad anti-apoptotic function of Hsp60 in cancer, which also includes restraining p53 activity, and promoting survivin stability (12). As a regulator of CypD in tumors, Hsp60 assembles in a multi-chaperone complex, comprising also at least Hsp90 and TRAP-1 (6). This mitochondrial chaperone network is required for tumor maintenance, as silencing of Hsp60 (this study), or inhibition of Hsp90 or TRAP-1 activity (9) triggered CypD-dependent permeability transition, apoptosis, and suppression of tumor growth, *in vivo* (9). In contrast, Hsp60 (12) or Hsp90 (9) targeting had no effect on normal cells, consistent with the low levels of these chaperones in mitochondria, and their lack of physical interaction with CypD. Altogether, these data suggest that regulatory pathways of mitochondrial permeability transition may be *qualitatively* different in tumor *versus* normal mitochondria, and thus provide unique molecular targets for novel cancer therapeutics (9).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Chaperone-CypD network in tumor mitochondria

A, Mitochondrial extracts from HCT116 cells or normal mouse liver were fractionated by FPLC and an elution profile was recorded. AU, absorbance units. B, The indicated FPLC fractions were analyzed by silver staining. C, D, FPLC fractions eluted from HCT116 cells (C) or normal liver (D) mitochondria were analyzed by Western blotting.

Figure 2. Hsp60-CypD complex

A, HCT116 mitochondrial extracts were mixed with the indicated GST fusion proteins, and pellets were analyzed by Western blotting. B, The indicated GST fusion proteins were incubated with recombinant Hsp60, and pellets were analyzed by Western blotting. Recombinant Hsp60 (rHsp60) was used as marker. C, Mitochondrial (Mito) or cytosolic (Cyt) extracts from HCT116 cells were immunoprecipitated (IP) with IgG or an antibody to Hsp60, and pellets (P) or supernatants (S) were analyzed by Western blotting. D, The indicated GST fusion proteins were incubated with extracts from normal human fibroblasts (NHF), and pellets were analyzed by Western blotting. *Right*, CypD expression in normal or tumor human cell lines. For A, B and D, bottom panels are Coomassie staining of recombinant proteins.

Figure 3. Hsp60 antagonizes CypD-mediated mitochondrial permeability transition

A, siRNA-transfected HCT116 cells with or without cyclosporine A (CsA) were analyzed by Western blotting. B, siRNA-transfected HCT116 cells were analyzed for propidium iodide (PI) staining and DEVDase (caspase) activity by multiparametric flow cytometry, in the presence or absence of CsA. The percentage of cells in each quadrant is indicated. C, LN229 cells stably transfected with control shRNA (pLKO) or CypD-directed shRNA (CypD) were analyzed by Western blotting. D, LN229 cells stably transfected with control (Ctrl) or CypD-directed shRNA were analyzed for cell viability by MTT. Mean±SEM. **, $p=0.002$.

Figure 4. Hsp60 requirement for tumor growth, *in vivo*

A, U87-Luc glioblastoma cells stably transfected with scrambled or Hsp60-directed shRNA were analyzed by Western blotting (*left*) or cell proliferation by direct cell counting (*right*). Mean $\pm$ SEM. B, shRNA transfected U87-Luc cells were stereotactically injected in the right cerebral striatum of immunocompromised mice, and tumor growth was monitored at the indicated time intervals by bioluminescence imaging (*top*) and quantified (*bottom*). C, Animal survival in scrambled or Hsp60 knockdown group was quantified by a Kaplan-Meier curve. $p < 0.05$. D, Tumor samples from scrambled or Hsp60 shRNA knockdown group were harvested at the end of the experiment and analyzed with non-binding IgG or an antibody to Hsp60 by immunohistochemistry. BLU, bioluminescence units.