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Hypoxia death stimulus induces translocation of p53 protein to mitochondria

Detection by immunofluorescence on whole cells

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Abstract Evidence suggests that p53 induces cell death by a dual mode of action involving activation of target genes and transcriptionally independent direct signaling. Mitochondria are major signal transducers in apoptosis. We recently discovered that a fraction of induced p53 protein rapidly translocates to mitochondria during p53-dependent apoptosis, but not during p53-independent apoptosis or p53-mediated cell cycle arrest. Importantly, specific targeting of p53 to mitochondria was sufficient to induce apoptosis in p53-deficient tumor cells. This led us to propose a model where p53 exerts a direct apoptogenic role at the mitochondria, thereby enhancing the transcription-dependent apoptosis of p53. Here we show for the first time that mitochondrial localization of endogenous p53 can be visualized by immunofluorescence of whole cells when stressed by hypoxic conditions. Suborganellar localization by limited trypsin digestion of isolated mitochondria from stressed cells suggests that a significant amount of mitochondrial p53 is located at the surface of the organelle. This mitochondrial association can be reproduced in vitro with purified p53. Together, our data provide further evidence for an apoptogenic signaling role of p53 protein in vivo at the level of the mitochondria.

Key words: p53; Mitochondrial localization; Apoptosis; Hypoxia; Immunofluorescence

1. Introduction

Despite its central role in cell death, the mechanism of p53-mediated apoptosis after cellular stress remains unclear. Current evidence indicates that p53 induces cell death by a complex dual mode of action involving transactivation of target genes and direct signaling events which are transcription-independent (reviewed in [1,2]). With respect to the transactivation mode, which is the better studied, a growing and diverse list of p53-inducible genes are candidates for participating in p53's apoptotic action. These include target genes with proven roles in apoptotic pathways such as the death receptors *Fas/APO-1* [3,4] and *KILLER/DR5* [5] and target genes whose products act directly on mitochondria such as *Bax* [6], Noxa [7] and p53 apoptosis-inducing protein 1 [8]. In addition, genes with less well defined apoptotic roles such as the PIG redox-modulators [9] and the *p85* regulatory subunit of phosphatidyl-3-OH kinase [10] have also been proposed. This array of potential targets likely have cell type and death stimulus specificity. It is also in sharp contrast to the few response genes through which p53 mediates cell cycle arrest (e.g. Waf1 and 14-3-3s) and indicates that p53 does not rely on any one of these downstream effectors alone to exert its powerful death role.

Evidence for an additional transcription-independent pathway for p53-mediated apoptosis is accumulating. In some cell types, p53-dependent apoptosis occurs in the absence of gene transcription or protein synthesis [11–13] or upon expression of the transcriptionally inactive mutants p53 (1-214) [14,15] and p53 Gln-22,Ser-23 [16]. In addition, a tumor-derived p53 mutant exists (p53 R175P) that transactivates p53 targets involved in cell cycle arrest but is defective for apoptosis [17]. This indicates that the transactivation activity of p53 is not sufficient for an apoptotic response and suggests that p53 can induce apoptosis through a transcription-independent mechanism. In vascular smooth muscle cells, p53 protein can mediate apoptosis by directing Fas redistribution from the Golgi complex to the cell surface [18]. Most interestingly, p53 protein from cell-free postnuclear cytosolic extracts which still contain mitochondria mediates the activation of effector caspases in cells undergoing p53-dependent apoptosis [19]. This indicates a transcription-independent p53 action and suggests protein–protein signaling from p53 to the Casp9/Casp3 cascade.

Mitochondria are central integrators and transducers for pro-apoptotic signals. This is particularly but not exclusively the case with those inducers of cell death that activate apoptosis independently of death receptor pathways. Such inducers include cell damage from g-IR, anticancer drugs, hypoxia and growth factor withdrawal. A major reason for the central role of mitochondria is that these organelles store critical apoptotic activators and effectors of cell death in their intermembranous space. These include cytochrome *c*, Smac/Diablo (a caspase co-activator), apoptosis-inducing factor which activates nuclear endonucleases and procaspases 2 and 9 ([20–23]; reviewed in [24,25]). Permeabilization of mitochondrial membranes causes the release of these activators and triggers the killing of cells.

Recently we showed that a fraction of induced p53 protein directly translocates to mitochondria in response to death signals from anticancer drugs [26]. This translocation is spe-

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cific for p53-dependent apoptosis but not for p53-independent apoptosis or for p53-dependent arrest and occurs in a variety of non-malignant and malignant human and mouse cells. Induced p53 translocation is rapid (1 h) and precedes changes in mitochondrial membrane potential, cytochrome *c* release and procaspase 3 activation. Ectopic overexpression of anti-apoptotic bcl-2 or bcl-xL abrogates stress-induced p53 translocation to mitochondria and apoptosis but does not abrogate stress-induced cell cycle arrest. Most importantly, specific targeting of p53 to mitochondria is sufficient to cause cell death. The mitochondrial p53 action is transcription-independent since targeted mutant p53 R175H, which is completely inactive as a nuclear version, remains active as a mitochondrial protein. We therefore proposed that this apoptogenic mitochondrial action of p53 enhances the transcriptional action of p53 to mediate cell death [26].

Previously we used subcellular fractionation and immunoelectronmicroscopy of isolated mitochondria to show the stress-induced mitochondrial p53 translocation. The purpose of the current study was to substantiate these findings by direct visualization of mitochondrial p53 in whole cells, to test this mitochondrial p53 association in vitro and to identify the major suborganellar compartment of p53 localization. We find that short term hypoxia treatment provides a brief window in time before the protein accumulates in the nuclei, which is optimal for visualizing mitochondrial staining of p53 in whole cells. Moreover, we show that purified p53 specifically associates with mitochondria in vitro. Suborganellar localization studies using limited trypsin digestion suggest that the majority of mitochondrial p53 locates to the surface of mitochondria.

2. Materials and methods

2.1. Cell culture

Human ML-1 cells, harboring wild type (wt) p53, were grown in DMEM supplemented with 10% fetal bovine serum. Camptothecin (Sigma) was added to freshly seeded cultures. For hypoxic conditions, freshly seeded cells were placed into GasPak plastic pouches (BBL GasPak Pouch Systems, Becton Dickinson), tightly sealed and placed back into the incubator for the indicated times. The pouches contain an iron-based oxygen consuming chemical, an O_2 indicator strip and a carbon dioxide generator. As certified by the manufacturer, they generate a CO_2-enriched hypoxic microenvironment with an oxygen concentration of $<2\%$ and a CO_2 concentration of $>4\%$ within 2 h of incubation.

Alternatively, hypoxia conditions were generated by adding deferoxamine mesylate (DXE, Sigma) into the culture medium for the indicated times.

2.2. Preparation of mitochondrial fractions

Mitochondria were prepared by sucrose density gradients as we previously described [27]. Mitochondria were collected at the 1–1.5 M interphase by lateral suction, washed in four volumes of MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris pH 7.6, 5 mM EDTA), pelleted at 14 000 rpm for 10 min and carefully resuspended in a final volume of 200 μl MS buffer. Limited trypsin digestion of mitochondrial fractions and crude cell lysates (75 μg protein/50 μl MS buffer) was performed as described [28] at room temperature for 10 and 20 min using TPCK-treated trypsin (Sigma) at a ratio of 1:500 trypsin:total protein. For in vitro assays of mitochondrial p53 association, mitochondria from untreated ML-1 cells were freshly prepared by sucrose density gradients as above. Isolated mitochondria (37 μg total protein) were incubated with 60 ng of purified baculoviral human wt p53 (untagged, purified by sequential MonoQ columns to obtain a single band by silver gel), or with 60 ng of purified recombinant (*Escherichia coli*) human wt PCNA (6× His-tagged, purified by Ni-NTA metal affinity column, Qiagen) for 1 h at 37°C in MS buffer containing 150 μM $CaCl_2$. Mitochondria were pelleted (14 000 rpm for 10 min) and washed three times in 1 ml of MS buffer each prior to SDS–PAGE.

2.3. Immunofluorescence

ML-1 cells were either left untreated or hypoxia-treated for 6 h (GasPak pouches or 125 μM deferoxamine). 30 min before fixation, all cells were in vivo labeled by adding 50 nM Mito Tracker Red CMXRos (Molecular Probes) into the medium. To test for specificity of Mito Tracker staining, cells were pretreated for 10 min with the uncoupler FCCP (50 nM) (carbonyl cyanide *p*-(tri-fluoromethoxy)-phenyl-hydrazone) prior to labeling with Mito Tracker as above.

Cells were quickly cytospun onto glass slides, fixed for 3 min with ice cold 1:1 acetone/methanol, washed and blocked in 10% goat serum followed by CM-1 (rabbit anti-human p53, 1:500). After washing, cells were incubated with biotinylated goat anti-rabbit IgG followed by streptavidin-conjugated FITC (1:200 in blocking solution) (Zymed Labs, San Francisco, CA, USA) and mounted with glycerol-free SlowFade (Molecular Probes).

2.4. Immunoblot and apoptosis assays

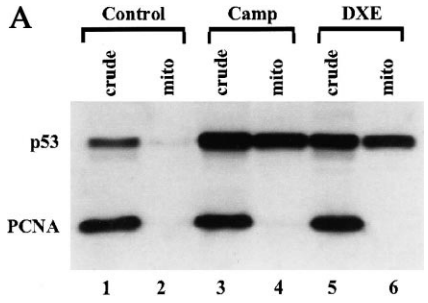
Equal amounts of total protein of mitochondrial or crude cell lysates (10–25 μg) were subjected to Western blot analysis and developed with enhanced chemiluminescence (Amersham) for 15–60 s. The following antibodies were used: monoclonal DO-1 (Calbiochem) for human p53; monoclonal mt hsp60 and mt hsp70 (Affinity Bioreagents); monoclonal PCNA (Santa Cruz); monoclonal cytochrome *c* (Pharmingen), cytochrome oxidase IV (Molecular Probes) and polyclonal bcl-xL (Santa Cruz). Apoptosis was assessed by the in situ TUNEL assay (Boehringer).

3. Results

3.1. Short term hypoxic stress induces mitochondrial p53 detectable by immunofluorescence of whole cells

We recently showed that a fraction of death signal-induced p53 localizes to mitochondria [26]. This phenomenon is widespread and occurs in human and mouse non-malignant and malignant cells in response to various types of stress such as DNA damage by various topoisomerase inhibitors (e.g. camptothecin). Here we further explore p53 localization to mitochondria in response to hypoxia in ML-1 cells. ML-1 cells are a human chronic myelogenous leukemia line harboring wt p53 which is functionally fully competent [26]. To this end, highly enriched mitochondrial fractions from ML-1 cells before and after apoptosis induction were prepared by classic discontinuous sucrose gradients [27] and analyzed by immunoblots. Mitochondrial fractions prepared in this fashion are over 90% pure by electronmicroscopy and have only minimal contamination by other subcellular fractions as we previously showed by marker studies (see [26]). Most importantly, they are almost completely devoid of nuclear contamination as assessed by PCNA (Fig. 1A,C). Fig. 1A shows that hypoxia generated by a high dose of the hypoximimetic deferoxamine (DXE) (250 μM for 8 h) induces a similar degree of mitochondrial p53 as does the DNA damaging drug camptothecin (5 μM for 8 h) (compare lane 2 with lanes 4 and 6). The total cellular p53 induction by DXE ('crude'), reflecting mainly nuclear p53, is somewhat less than after camptothecin damage (lanes 1, 4 and 6).

To directly localize p53 to mitochondria of early apoptosing cells, we performed indirect immunofluorescence. Our earlier attempts with ML-1 cells using 8 h exposure to camptothecin, which induces double strand breaks, or to high dose DXE, showed intense nuclear staining but did not reproducibly detect the proportionately low levels of mitochondrial p53 due to interference by cytoplasmic background and the intensely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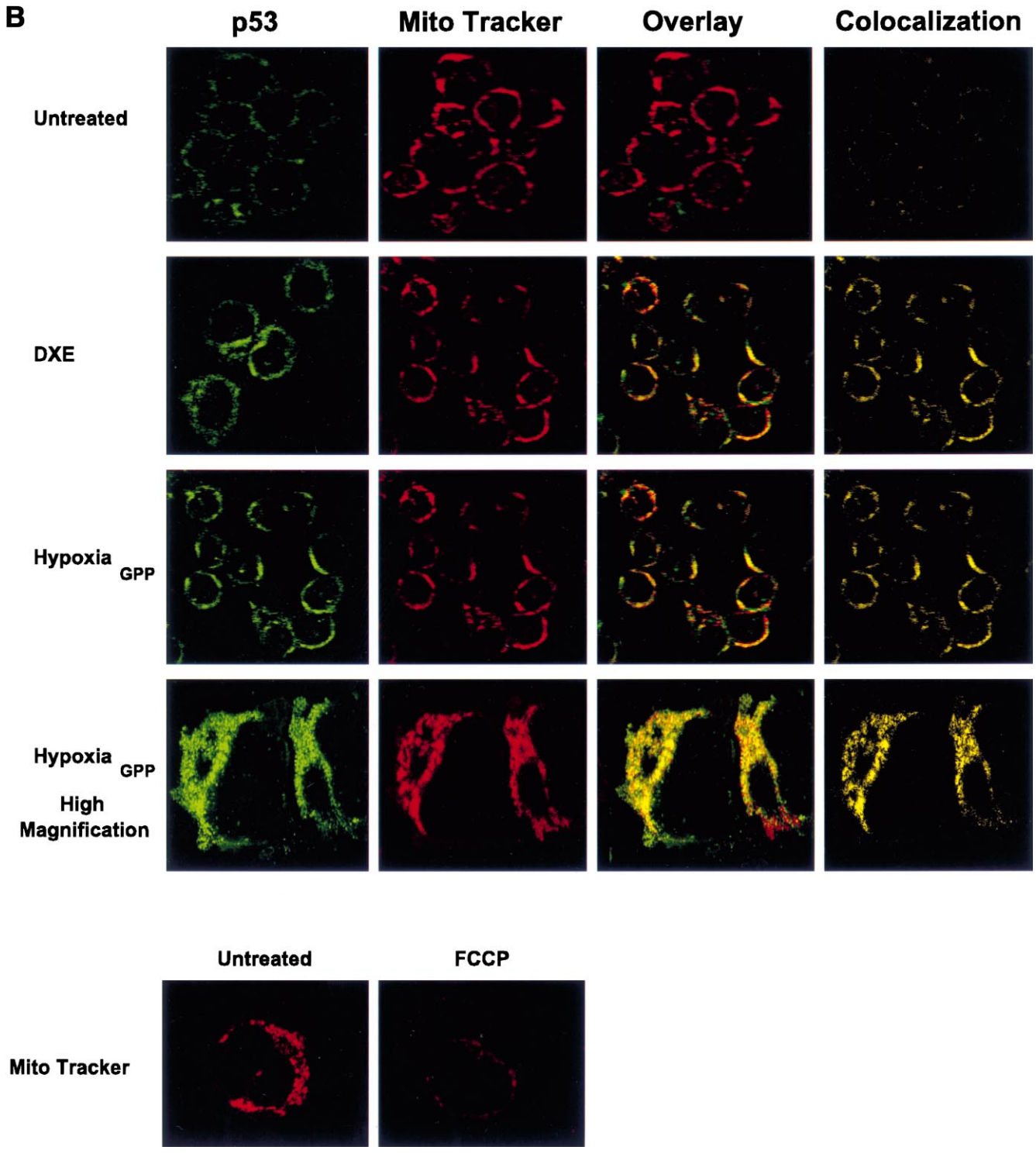
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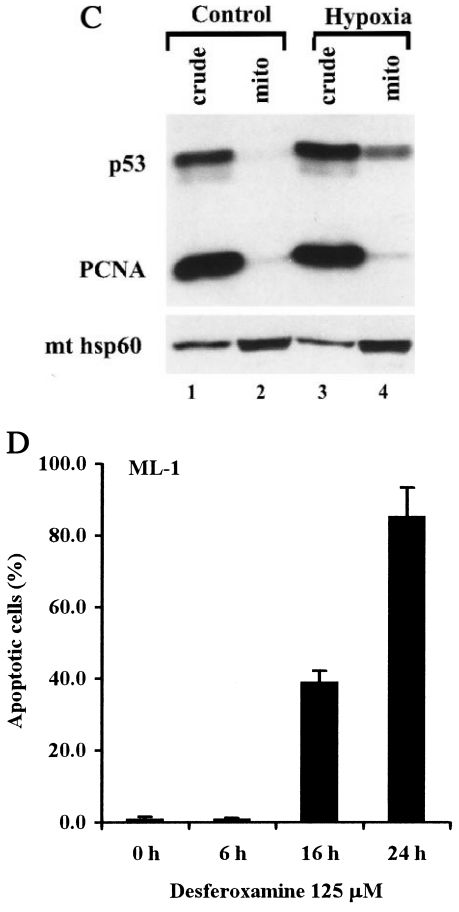

Fig. 1. A: Hypoxia generated by deferoxamine induces a similar degree of mitochondrial p53 as does DNA damage by camptothecin. Immunoblot of crude cell lysates and mitochondrial fractions before (C) and after treatment with camptothecin (5 μM, 8 h) or deferoxamine (250 μM, 8 h). PCNA is a marker for nuclear contamination. B: Short term hypoxic stress induces mitochondrial p53 detectable by immunofluorescence. Colocalization of p53 and mitochondria in the early phase of hypoxia. ML-1 cells untreated or after 6 h of hypoxia (125 μM deferoxamine or GasPak pouches GPP) were in vivo labeled with the mitochondria-specific potentiometric dye Mito Tracker, followed by immunofluorescence for p53 with CM-1. Confocal Nikon Diaphot 200 Inverted Microscope. All images were taken with identical acquisition parameters (laser intensity, photomultiplier, and Photoshop Software settings) at ×60 original magnification. The fourth row shows two cells captured at a resolution which visualizes individual mitochondria (zoomed in with Bio-Rad LaserSharp Software 2000). Colocalization is indicated in yellow. Bottom: when ML-1 cells were pretreated for 10 min with an uncoupler of oxidative phosphorylation (50 nM FCCP), Mito Tracker accumulation in mitochondria was greatly suppressed. C: Immunoblot of subcellular fractionation after short term hypoxia. A p53 band is detected in the mitochondrial fraction after 6 h of hypoxia (GasPak pouches). Control cells were left untreated. mt hsp60 is a mitochondrial and PCNA is a nuclear marker. D: Percentage of apoptosis of ML-1 cells induced by 125 μM deferoxamine after the indicated times. TUNEL assays.
←

staining large nuclei in these cells. By switching to milder hypoxic conditions (generated by hypoxic GasPak pouches or by lowering the dose of deferoxamine to 125 μM) and by decreasing the time of exposure, we determined that a narrow window exists at 6 h after treatment initiation when ML-1 cells are showing a punctate cytoplasmic p53 staining but have not yet induced strong nuclear staining, which would interfere with the narrow rim of cytoplasmic staining (Fig. 1B, left column, compare treated with untreated conditions). This punctate staining shows significant colocalization with mitochondria (Fig. 1B, yellow fluorescence). The requirement for optimized timing and stress conditions might explain why mitochondrial p53 escaped previous detection. To independently confirm mitochondrial localization of p53, we repeated the subcellular fractionation after 6 h of mild hypoxia and again could detect a p53 band in the mitochondrial fraction after treatment (Fig. 1C, compare lanes 2 and 4). Both modes of hypoxia (125 μM DXE or GasPak) induced the transcriptional activity of p53 as seen by p21 upregulation after 16 h (data not shown and [29]). TUNEL assays showed that hypoxia treatment of ML-1 cells induced greater than 80% cell death after 24 h of 125 μM deferoxamine (Fig. 1D). Together with the induction of cellular p53 (Fig. 1A,C), this indicates that hypoxia-mediated apoptosis in ML-1 cells has a significant p53-dependent component.

3.2. *The majority of mitochondrial p53 is located on the surface of the organelle*

Using isolated mitochondria we previously showed by immunogold electronmicroscopy that the majority of the stress-induced mitochondrial p53 appears to be located on the surface of the organelle, while a small subfraction formed an in vivo complex with the import protein mt hsp70 [26]. To support this result biochemically, we performed a classical mitochondrial import assay using limited protease digestion. After subjecting mitochondrial isolates from stressed cells to controlled trypsin digestion which removes proteins attached to the outside of the organelle [30], the majority of the associated p53 protein was digested after 10 min, as indicated by the drastic decrease in band intensity of full length p53 and the novel appearance of a cleaved p53 fragment (Fig. 2, compare lane 2 with lane 4). Bcl-xL protein, located on the outer mitochondrial membrane, served a positive control for this assay. Bcl-xL was digested with similar kinetics as p53. In contrast, cytochrome *c*, which resides in the intermembranous

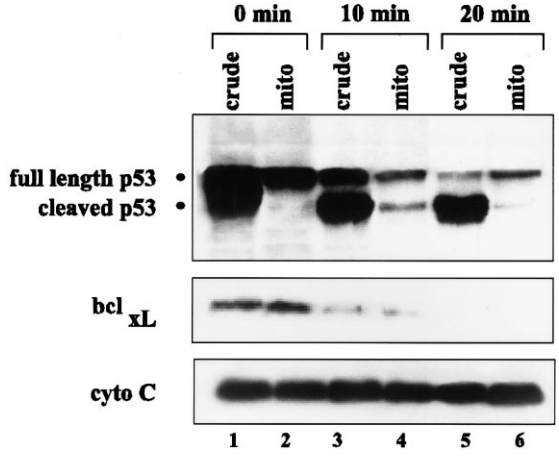

Fig. 2. The majority of mitochondrial p53 locates to the surface of the organelle. Trypsin digestion of isolated mitochondria treated with camptothecin (5 mM for 5 h). Immunoblot of digests after 0, 10 and 20 min probed with specific antibodies for p53, bcl-xL and cytochrome *c*. The majority of mitochondrial p53 is unprotected and cleaved after 10 min, as is the outer membrane protein bcl-xL, while a small portion is protected, suggesting import (lanes 4 and 6). The intermembranous protein cytochrome *c* is protected.

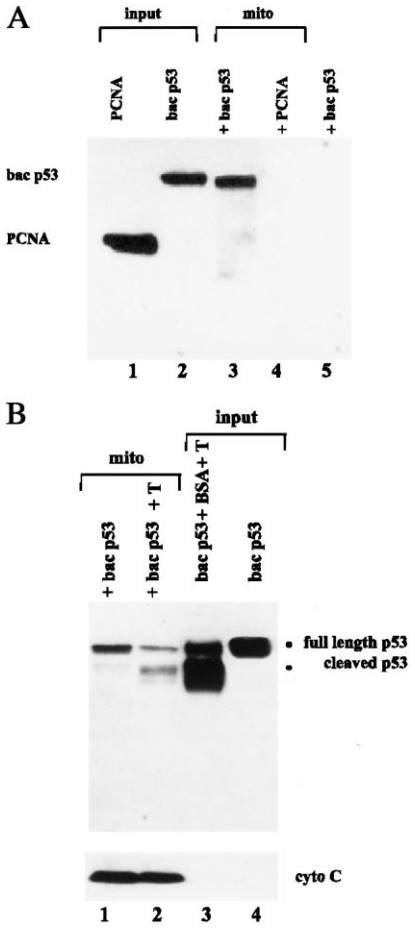

Fig. 3. In vitro localization assay of p53. A: Isolated mitochondria (37 mg total protein) from ML-1 cells were incubated at 37°C for 1 h with highly purified (silver gel pure) untagged baculoviral human wt p53 or recombinant PCNA (60 ng each). Proteins associated with mitochondria were recovered by pelleting, followed by three washes in MS buffer (lanes 3 and 4). Lane 5 is identical to lane 3 except that mitochondria were omitted, confirming that bac p53 cannot precipitate by itself. As control, 1/10 of the input aliquots was loaded directly onto the gel (lanes 1 and 2). B: In vitro associated mitochondrial p53 before (lane 1) and after trypsin digestion (lane 2). Conditions as in Fig. 2. As control, 1/5 of the input aliquots before (lane 4) and after (lane 3) trypsin digestion was loaded directly onto the gel. Bovine serum albumin (BSA) is used to substitute for mitochondrial proteins.

space and therefore should be protected from trypsin, was not digested, thus validating the assay conditions. A small fraction of p53, however, remained protected even after 20 min digestion (compare lanes 2 with 4 and 6), indicating intraorganellar location of a small subfraction of mitochondrial p53. This might represent the subfraction of mitochondrial p53 engaged in the in vivo complex with mitochondrial mt hsp70 protein which we previously described [26].

3.3. p53 associates with mitochondria in vitro

The association of p53 with mitochondria was also reproducible in vitro (Fig. 3A). When intact mitochondria isolated from ML-1 cells were incubated with purified baculoviral human wt p53 or recombinant human PCNA protein (lanes 1, 2), only p53 but not PCNA became associated with mitochondria, as determined by co-sedimentation with the organelles (lanes 3, 4). Mock incubation without mitochondria ruled out self-precipitation of baculoviral p53 (lane 5). Moreover, the associated p53 was largely trypsin sensitive, but again showed a small amount of protected p53 (Fig. 3B, lanes 1 and 2), thereby recapitulating the situation already seen with endogenous mitochondria (Fig. 2).

4. Discussion

Our data show that a fraction of induced p53 protein specifically localizes to mitochondria at the onset of hypoxia-mediated apoptosis. In addition to true hypoxia, a condition which frequently exists within solid tumors, we show that the chemical hypoximimetic deferoxamine [28] also induces targeting of endogenous p53 to mitochondria. DXE induces apoptosis [31–33]. It acts within the O_2 tension signal transduction pathway [34] by chelating ferrous iron and might function by displacing ferrous iron from the porphyrin ring of the putative mammalian hemoprotein O_2 sensor [35]. DXE is a hypoximimetic because it activates hypoxia-inducible transcription factor 1α activity (HIF-1α) and stimulates transcription of hypoxia-associated genes including VEGF and erythropoietin [32,35,36]. However, the mode of action differs between true hypoxia and DXE. Evidence suggests that mitochondrial ROS are necessary and sufficient for stabilization of HIF-1α during true hypoxia (1% O_2). In contrast, DXE responses require neither the mitochondria nor ROS, suggesting that DXE acts at a more distal step in the hypoxia signaling pathway [37]. Importantly, DXE induces transcriptionally active p53 in a HIF-1α-dependent fashion, possibly via direct complex formation between p53 and HIF-1α proteins [32].

This report is the first direct in vivo demonstration of the targeting response of endogenous mitochondrial p53. Mitochondrial p53 translocation can be visualized by immunofluorescence at 6 h on whole cells, before p53 has reached its maximum accumulation in the nucleus (see Fig. 1B). This timeframe is consistent with our previous subfractionation results after camptothecin, where mitochondrial p53 is highest after 6 h [26]. Moreover, the in vivo targeting response could be reproduced in vitro with isolated mitochondria and purified p53, suggesting that p53 might bind to protein component(s) at the mitochondrial surface. In keeping with this notion, our trypsin data suggest that p53's main location in mitochondria is on the surface. Limited digestion of isolated mitochondria from stressed cells (Fig. 2) as well as from mitochondria bound to purified p53 in vitro (Fig. 3B) shows that the majority of mitochondrial p53 is digested with kinetics similar to bcl-xL, an outer membrane-associated anti-apoptotic bcl member. The relationship of the surface-associated p53 and the subfraction of mitochondrial p53 that complexes with mt hsp70 in vivo [26] will require further investigation. One possibility is that p53 has an additional intraorganellar compartment which involves a transient import step by mt hsp70, followed by subsequent resorting to the final compartment. Interestingly, an amphipathic N-terminal import leader peptide is not essential for import, as indicated by cytochrome *c* which is located at the outer face of the inner membrane and also lacks this sequence [38].

Following our initial description [26], a second nuclear transcription factor, the orphan nuclear steroid receptor TR3 (also called Nur77 or NGFIB), was also found to translocate to mitochondria in response to a wide variety of pro-apoptotic signals where it triggers membrane permeability and apoptotic

cell death. As is true for mitochondrial p53, mitochondrial TR3 is sufficient to cause cell death and its action is blocked by bcl-2 [39]. Thus, there might be now two nuclear transcription factors that, by virtue of their subcellular relocalization, are capable of mediating lethal signaling directly through mitochondria [40]. In the case of p53, its mitochondrial action likely enhances the transcriptional action of nuclear p53 to mediate cell death.

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