The mechanism of p53-mediated apoptosis after cellular stress remains poorly understood. Evidence suggests that p53 induces cell death by a multitude of molecular pathways involving activation of target genes and transcriptionally independent direct signaling. Mitochondria play a key role in apoptosis. We show here that a fraction of p53 protein localizes to mitochondria at the onset of p53-dependent apoptosis but not during p53-independent apoptosis or p53-mediated cell cycle arrest. The accumulation of p53 to mitochondria is rapid (within 1 h after p53 activation) and preceeds changes in mitochondrial membrane potential, cytochrome c release, and procaspase-3 activation. Immunoelectron microscopy and immuno-fluorescence-activated cell sorter analysis of isolated mitochondria show that the majority of mitochondrial p53 localizes to the membranous compartiment, whereas a fraction is found in a complex with the mitochondrial import motor mt hsp70. After induction of ectopic p53 without additional DNA damage in p53-deficient cells, p53 again partially localizes to mitochondria, preceeding the onset of apoptosis. Overexpression of anti-apoptotic Bcl-2 or Bcl-XL abrogates stress signal-mediated mitochondrial p53 accumulation and apoptosis but not cell cycle arrest, suggesting a feedback signaling loop between p53 and mitochondrial apoptotic regulators. Importantly, bypassing the nucleus by targeting p53 to mitochondria using import leader fusions is sufficient to induce apoptosis in p53-deficient cells. We propose a model where p53 can contribute to apoptosis by direct signaling at the mitochondria, thereby amplifying the transcription-dependent apoptosis of p53.
permeability transition pore opening, and mitochondrial swelling with outer membrane rupture in some instances. One critical consequence of all these mitochondrial alterations is the rapid release of caspase activators such as cytochrome c from the intermembranous space into the cytosol. Released cytochrome c is the penultimate trigger for caspase-3 activation via the Apaf-1 pathway (for review see Refs. 28 and 30). Bcl-2 and Bcl-xL family members, which reside in the outer mitochondrial membrane, protect cells from death largely by antagonizing mitochondrial dysfunction, preventing all hallmarks of the early stages of apoptosis. They counteract abnormal ion movements, thereby stabilizing the inner mitochondrial transmembrane potential Δψ and mitochondrial volume and prevent the release of apoptogenic protease activators (28, 30, 31).

Based on the transcription-independence actions of p53 and the importance of mitochondria in p53-dependent apoptosis (17), we investigated the possibility that, in addition to its transactivation role in the nucleus, p53 might have a role at this extranuclear regulatory organelle. Surprisingly, we found that a small but highly reproducible fraction of stress-induced p53 protein traffics to mitochondria at the onset of p53-dependent apoptosis after either DNA damage or hypoxic damage. This effect does not occur during p53-independent apoptosis or during p53-mediated cell cycle arrest. The localization of p53 to mitochondria is rapid, preceding the release of cytochrome c and procaspase-3 activation and is blocked by overexpression of anti-apoptotic Bcl proteins. Importantly, redirecting p53 from the nucleus and targeting it to mitochondria by using mitochondrial import leader peptides is sufficient to induce apoptosis in p53-deficient SaOs-2 cells. These findings support a novel role of the p53 protein in direct apoptotic signaling at mitochondria, which are a strategic point in the cell death cascade. The action of p53 at the mitochondrial site appears to be a significant enhancer to its transactivation mode in the nucleus, thereby amplifying its apoptotic potency.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—ML-1, RKO, MCF-7, IMR 90, MRC 5, and EB-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. 32D lines and Ba f-3 cells were grown in RPMI 1640, 10% fetal bovine serum supplemented with IL-3 (20 units/ml) and 10% fetal bovine serum. 32D lines and Baf-3 cells were grown in RPMI 1640, 10% fetal bovine serum supplemented with IL-3 (20 units/ml). Actinomycin D; TUNEL, Tdt-mediated dUTP-X nick end labeling; IL-3, interleukin-3; PCNA, proliferating cell nuclear antigen; Camp, camptothecin; wt, wild type; Rh, retinobiolastoma.

1 The abbreviations used are: TNF-α, tumor necrosis factor-α; mt, mitochondrial; Act D, actinomycin D; TUNEL, Tdt-mediated dUTP-X nick end labeling; IL-3, interleukin-3; PCNA, proliferating cell nuclear antigen; Camp, camptothecin; wt, wild type; Rh, retinobiolastoma.

PAb 1801, DO-1 (Calbiochem), and polyclonal CM-1 (Vector) for human p53, and CM-5 (gift of D. Lane) for mouse p53; monoclonal mt hsp70 (Affinity Bioreagents) and polyclonal Grp75 (gift of B. Merrick) for mthsp 70 (neither cross-reacts with mt hsp60 or cytosolic heat shock proteins); monoclonal Rb, PCNA, Ld3a (Santa Cruz Biotechnology); monoclonal Bax, Bcl-2, Bcl-xL, Lc3a, Bcl-60, GC3, and cytoplasmic GSP (Affinity Bioreagents); cytochrome c (PharMingen) and cytochrome oxidase IV (Molecular Probes); polyclonal BCL-2 and BCL-XL (Santa Cruz Biotechnology), and monoclonal FLAG (Kodak) or preimmune rabbit IgG (Sigma) for negative controls. For determining the percentage of mitochondrial p53 relative to total induced p53, DO-1 blots of serial dilutions were incubated with anti-mouse IgG coupled to alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Kirkegaard & Perry Laboratories) and quantitated by densitometry (Bio-Rad). Mitochondrial fractions (70 μg per sample) from treated ML-1 cells (5 μM Camp for 6 h) were immunoprecipitated with 1 μg of antibody and immunoblotted for co-precipitating proteins. Apoptosis was assessed by the sub-G1 fraction of propidium iodide-labeled cells (2 μg/ml, Sigma) on a FACScan (Becton Dickinson) (10,000 events each). For morphologic confirmation, electron microscopy was done. Alternatively, the in situ TUNEL assay (Roche Molecular Biochemicals) or Hoechst 33342 staining (50 μg/ml, Sigma) was performed. For p53-independent apoptosis, ML-1 cells were incubated with TNF-α (30 ng/ml) and Act D (0.7 μm) (33). After 16 h, mitochondrial fractions were prepared, and apoptosis was determined by TUNEL.

**Flow Cytometry of Isolated Mitochondria**—Freshly isolated (non-permeabilized) mitochondria from untreated and treated ML-1 cells (1 × 10^6 cells) were incubated with the indicated antibodies (1 μg/ml) for 15 min on ice, washed, and stained with secondary fluorescein isothiocyanate-conjugated anti-mouse IgG (1 μg/ml) (Life Technologies, Inc.) for 15 min. Mitochondria were then again washed, resuspended in MS buffer, and gated for non-aggregated particles on a FACSscan as described (34). To measure mitochondrial membrane potential, whole cells were incubated in Rhodamine 123 (0.2 μg/ml) (Molecular Probes) added to the culture for 30 min at 37 °C, washed, and resuspended in propidium iodide to gate on viable cells by FACSscan (34).

Quantitative Immunofluorescence of Isolated Mitochondria—Aliquots of isolated mitochondria from treated ML-1 cells (5 μM Camp for 5 h) were applied to chamber slides (Nunc) and immunostained as described above. For each antibody standardized epifluorescent images were acquired as follows: an individual from the Imaging Center, without knowledge of the antibody identity, acquired three random fields without prior visual selection at identical camera settings. Raw data for each triplet were then processed to measure two independent parameters: staining intensity, mean fluorescent pixel intensity and total pixel area (MetaMorph Program). The control antibody (FLAG) was normalized to 100%.

**Immunocytochemistry—Treated (5 μM Camp for 5 h) and untreated mitochondrial fractions of ML-1 cells were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde for 20 min at room temperature and embedded in LR White resin under UV cross-linking on slides with mouse antibodies (10 μg/ml) followed by sheep anti-mouse IgG conjugated to 12 nm colloidal gold (Jackson ImmunoResearch) and viewed under a Zeiss EM 10. Secondary antibody alone was run as control. Pellets showed 90% purity for mitochondria as identified by double membranes and cristae. For quantitation, see "Results."
p53 Protein Partially Accumulates on Mitochondria During p53-dependent Apoptosis

(38). Expression was reproducibly about 30%, and similar among all constructs as judged by FLAG intensity. For each construct, FLAG-positive cells (total number between 200 and 600 cells depending on the construct) and TUNEL-positive cells from duplicate chambers were counted in 20 random fields each and the percentage of apoptosis of FLAG-positive cells in non-malignant and malignant cells was determined after correction for background with vector alone (~2%). Experiments were performed 4–8 times, depending on the construct.

RESULTS

In Vivo Correlation between Induction of Mitochondrial p53 and p53-mediated Apoptosis in a Variety of Cell Types and Cell Stresses—Based on evidence for the transcription-independent action of p53 in cell death and the central importance of mitochondria in this process, we investigated a possible link in which p53 might have an additional role at this extranuclear site. To this end, highly enriched mitochondrial fractions from cells before and after apoptosis induction were prepared by classic discontinuous 1–2 μ sucrose gradients (32), washed in mitochondria stabilizing buffer, and analyzed by immunoblots along with other cellular fractions. In parallel, apoptosis was measured by cell cycle analysis or TUNEL assay and confirmed by Hoechst staining or electron microscopy morphology (e.g. Fig. 1, A and B, and data not shown).

Mitochondrial fractions from two human cell types (both harboring wild type p53), the myeloid leukemia line ML-1 (Fig. 1A, lanes 5 and 2, and Fig. 1B, lanes 2 and 4) and the colorectal carcinoma line RKO (Fig. 1C, lanes 5 and 3) were compared before and after apoptosis induction by DNA damage using 5 μM camptothecin (Fig. 1, A and C) or before and after apoptosis induction by hypoxic stress using 250 μM desferoxamine (Fig. 1B). In both cell lines, a portion of p53 rapidly localized to the mitochondrial fraction of apoptosing cells. Identical results for both drugs were also seen with the human breast cancer cell line MCF-7 (wild type p53) (data not shown). Moreover, identical results were found in mouse 32D cells (immortal myeloid progenitors containing wild type p53) (Fig. 3C, lanes 2 and 4) and in Baf-3 cells (preB-cells with wild type p53) (data not shown), which undergo p53-dependent apoptosis after DNA damage plus IL-3 withdrawal (39). Although mitochondrial p53 comprised only a small fraction of induced cellular p53, it was a highly reproducible observation (in all of over 60 experiments). In ML-1 cells, we measured about 2% of total induced p53 after camptothecin treatment, as quantitated by densitometry of alkaline phosphatase-based p53 immunoblots (data not shown). Mitochondrial accumulation was specific for p53, since the other nuclear proteins such as Rb and the highly abundant PCNA did not accumulate (Fig. 1, A–C). Mitochondrial fractions were also stringently monitored for contamination by using markers for the nucleus (PCNA and Rb), cytoplasm (IxB), endoplasmic reticulum (BiP), and Golgi (βCOP), which were either absent (Rb, PCNA, and IxB) or barely detectable (BiP and βCOP) (Fig. 1, A–C). In the case of BiP and βCOP, the small amounts present in the untreated mitochondrial fractions further decreased to trace amounts in the treated mitochondrial fractions. In addition, mitochondrial fractions were examined by conventional electron microscopy and found to be >90% pure (double contoured intact organelles with cisternae), with the remaining consisting of lysosomes and membrane fragments. Thus, mitochondrial p53 accumulation is a widespread phenomenon that occurs in vivo during various types of stress responses and across species was demonstrated after malignant cells.

p53 Protein Does Not Localize to Mitochondria During p53-independent Apoptosis or during p53-mediated Cell Cycle Arrest—If the above phenomenon is potentially of physiologic significance, it should be restricted to p53-dependent apoptosis. To test this hypothesis, we subjected ML-1 cells to p53-independent apoptosis by the TNF pathway. Mitochondria are a downstream component of the TNF-R/DISC/caspase-8 cascade (for review see Ref. 29). In contrast to our findings in p53-dependent cell death, p53 did not accumulate to mitochondria when ML-1 cells were induced to undergo p53-independent death by the TNF pathway. Fig. 1D shows that mitochondrial fractions from ML-1 cells treated with TNF-α plus actinomycin D do not contain significant levels of p53, despite induction of total cellular p53 levels and a degree of apoptosis that was similar to the one seen with for example desferoxamine (compare with Fig. 1B). These data indicate that mitochondrial p53 association does not represent a nonspecific event of high cellular p53 levels or damaged organelles nor is it a result of cells undergoing cell death. Instead, the data strongly suggest that mitochondrial p53 is part of a regulatory response that governs a p53-dependent apoptotic outcome.

Moreover, fibroblasts, which are prototypical for cell types whose intrinsic response to a wide variety of DNA damage is the activation of G1/G2 arrest checkpoints rather than apoptosis, fail to induce the accumulation of mitochondrial p53. In contrast to ML-1 cells, the immortal human diploid fibroblast lines IMR90 and MRC5, harboring wild type p53, failed to accumulate p53 in mitochondria after camptothecin (Fig. 1E) or etoposide (data not shown), concomitant with a lack of cytochrome c release (Fig. 1E). As expected, the strong induction of total cellular p53 (compare lanes 1 and 3) mediated a G1 arrest with decreasing S-phase from 40 to 22% (camptothecin) and 13% (etoposide) but no apoptosis. These data lend further specificity and physiologic significance to mitochondrial p53 accumulation during apoptosis but not during cell cycle arrest, which is governed by the transactivation activity of p53.

Mitochondrial p53 Accumulation Is Rapid, Preceding Changes in MΔψ, Cytochrome c Release, and Procaspase-3 Activation—To characterize this response in more detail, time course studies showed that mitochondrial fractions from ML-1 cells exposed to a death signal (5 μM Camp) started to accumulate p53 after only 1 h and continued to rise until 6 h, parallel to the kinetics of nuclear (crude) p53 induction (Fig. 2A). Subsequently, between 3 and 4 h, cytochrome c was released from the intermembranous space to trigger procaspase-3 activation, as indicated by the appearance of cleaved active enzyme after that time (Fig. 2A). Concomitantly, flow cytometric profiles of Rhodamine 123 fluorescence on whole cells after in vivo uptake of the Rhodamine 123 lipophilic potentiometric dye showed early dysfunctional changes in the mitochondrial membrane potential MΔψ. Changes in MΔψ are known to appear well before the cells undergo apoptotic death (34). They consisted of early hyperpolarization and depolarization (34), as indicated by a lower and broader main peak and a second peak of low intensity that became detectable at 4.5 h (Fig. 2A, bottom, dark lines) when compared with untreated ML-1 cells (dotted lines). This dysfunctional profile became fully pronounced after 24 h as indicated by two mitochondrial subsets (Fig. 2A, bottom). Together, these data show that the kinetics of mitochondrial p53 localization precedes mitochondrial membrane changes, cytochrome c release, and procaspase-3 activation. We therefore conclude that mitochondrial p53 accumulation is an early event, again indicative of an active process, and not merely a consequence of apoptosis or a consequence of binding to damaged organelles in dying cells.

The Majority of p53 Locates to the Mitochondrial Membrane Compartment, Whereas a Fraction Is Found within the Organelle—To localize directly p53 to mitochondria of early apoptosing cells, we chose several independent approaches. Early studies with camptothecin-treated ML-1 cells showed that indirect immunofluorescence of whole cells primarily showed intense nuclear staining but did not convincingly detect the pro-
portionately low levels of mitochondrial p53 due to interference by diffuse cytoplasmic background staining. This could also explain why mitochondrial p53 localization escaped previous detection. We therefore used several alternative techniques that together clearly demonstrate mitochondrial p53 localization in early apoptosis.
Fig. 2. Kinetics and localization of mitochondrial p53. A, p53 accumulation on mitochondria precedes cytochrome c release and pro-caspase-3 activation. Immunoblots (5 μg of protein per lane) of mitochondrial fractions (mito) from ML-1 cells before and up to 6 h after adding 5 μM Camp. Cytochrome oxidase IV (cyto ox IV) validates equal mitochondrial loading. PCNA is used to verify the absence of significant nuclear p53 protein.
First, we performed flow cytometry analysis of mitochondria from ML-1 cells before and after DNA damage, isolated by sucrose density gradient and immunostained for p53 or control antibodies, as described previously (34). Fig. 2B shows that mitochondria from untreated ML-1 cells (dotted lines in all panels) bound the two different p53 monoclonal antibodies (1801 and DO-1) to the same low extent as they bound the monoclonal antibody to cytochrome oxidase IV (a marker protein of the inner mitochondrial membrane and not accessible in untreated, intact mitochondria) and an irrelevant control antibody (monoclonal FLAG). In contrast, early apoptosing mitochondria from ML-1 cells treated with 5 μM Camptothecin for 5 h (dark lines in all panels) showed enhanced and specific staining with both p53 antibodies, as indicated by a right shift in their fluorescence intensity profiles but not with the FLAG antibody (Fig. 2B). These data suggested p53 localization within the membranous compartment. The specificity of the p53 staining was further demonstrated by the newly appearing signal for cytochrome oxidase IV, which after treatment becomes accessible to its antibody due to mitochondrial swelling and rupture of the outer membrane (34) (Fig. 2B). These results were confirmed when the p53 signals from isolated apoptosing mitochondria were pixel-quantitated after 1801 immunostaining as described under “Experimental Procedures.” Table I shows that the p53 pixel intensity and area is significantly above the FLAG control (arbitrarily set as 100%) and resembles the pixel values received for the positive control antibody cytochrome oxidase IV.

Direct visual p53 localization was demonstrated by immunogold electron microscopy of apoptosing mitochondria. Washed mitochondria from treated (5 μM Camp for 5 h) and untreated ML-1 cells were isolated as before (>90% purity by EM), processed in LR White resin, and ultrathin sections were stained with either p53 antibody (DO-1) or mouse IgG at the same concentration (10 μg/ml). Staining was quantitated by randomly selecting 75 fields at low power (×2500) and then counting all mitochondria-associated gold grains, which became visible only at high power (×25,000) (Table II and Fig. 2C). p53-stained mitochondria from DNA-damaged cells yielded significant labeling (627 grains), with grains mainly decorating the mitochondrial membrane compartment, although some grains were also located within the organelle. In contrast, only background counts (32–34 grains) were obtained with p53-stained mitochondria in the absence of DNA damage or with mouse IgG-stained mitochondria irrespective of DNA damage (Table II). Secondary antibody alone gave only background staining (data not shown). Since mitochondria were swollen due to processing, more detailed submembranous localization with respect to the outer or inner membrane or intermembranous space was not possible.

To investigate a possible mechanism for mitochondrial p53 association, we looked for a physical interaction between the major mitochondrial import protein mt hsp70 and p53, mt hsp70, also called Grp75, is the major translocation motor that pulls proteins into mitochondria and refolds them before they get sorted into their final compartments within the membranes or the matrix (40, 41). Co-immunoprecipitation assays showed a specific in vivo complex between p53 and mt hsp70 (Fig. 2D). p53 co-precipitated with both polyclonal and monoclonal antibodies to mt hsp70 (lanes 4–6) and vice versa (lane 1) from isolated mitochondria of treated ML-1 cells. This is consistent with a previous observation of p53 co-immunoprecipitating with mt hsp70 in HT1080 cells (42). No p53 complexes were seen with irrelevant control antibodies (lanes 2, 3, and 7), an antibody to cytosolic heat shock protein hsc70 (data not shown), or an antibody to cytochrome oxidase IV (inner mitochondrial membrane protein) (lane 8), confirming that p53 fails to nonspecifically “stick” to damaged mitochondria. This lack of “stickiness” also holds for mt hsp70, since it does not associate with the abundant PCNA (lane 5).

Taken together, our data provide direct evidence that a fraction of induced p53 protein specifically localizes to mitochondria early in p53-mediated apoptosis. Moreover, the data suggest that the main location of p53 in mitochondria is in the membranous barrier compartment, probably after a transient import step that involves mt hsp70 before it is delivered to this final destination.

Functional Significance of Mitochondrial p53 Accumulation in p53-mediated Apoptosis—To examine further the functional significance of mitochondrial p53 localization in p53-dependent apoptosis, three approaches were taken. First, the ability of certain cell types to “choose” between the two p53-mediated damage responses provides a powerful dissecting tool within the same cell. ML-1 cells undergo either apoptosis or G1 arrest depending on the type of genotoxic drug used. While a 6-h exposure of camptothecin or etoposide induced apoptosis, actinomycin D (a topoisomerase inhibitor in the nanomolar range) almost exclusively induced G1 arrest (Fig. 3B). Concomitantly, p53 became detectable in the mitochondrial fractions of ML-1 cells treated with camptothecin or etoposide (Fig. 3A, compare lanes 2 with 4 and 6) but not with Act D (lanes 8 and 10). The 1.8 M Act D concentration induces total cellular p53 levels comparable to 5 μM camptothecin and 10 μM etoposide (lanes 11–14), with strong nuclear p53 in over 90% of ML-1 cells (8).
and 84% G1 arrest (Fig. 3B). As expected, the absence of apoptosis after Act D was paralleled by an absence of mitochondrial cytochrome c release (compare lanes 2 and 8), in contrast to treatment with apoptosis-inducing drugs (lanes 4 and 6). Even at the highest dose tested (3.6 nM Act D), mitochondria failed to accumulate p53 (lane 10). This result confirms and expands the result we obtained with fibroblasts undergoing cell cycle arrest instead of death after DNA damage (Fig. 1E). Thus, within the same cell, apoptosis-inducing drug treatment is associated with mitochondrial p53 accumulation, while G1 arrest-inducing drug treatment is not, despite comparable total cellular p53 induction.

Second, we asked whether mitochondrial p53 accumulation is associated with and precedes the acquisition of apoptosis in p53-deficient cells upon induction of p53 expression. The EB1 system, a stable human colon carcinoma line, harbors a met-
allothionine-driven wild type p53 transgene, whereas the parental cells have a homozygous deletion of the p53 gene and are incompetent for p53-mediated apoptosis (43). The advantage of this system is that (i) the apoptotic response is extensive after p53 induction alone and has no need for additional DNA damage (which due to its pleiotropic effects could conceivably cause nonspecific cellular events), (ii) wild type p53 expression is tightly regulated without leakage in the uninduced state, and (iii) EB1 cells are classic for studying p53-mediated apoptosis (43). As expected, uninduced EB1 cells lacked detectable p53 expression in crude cell extracts (Fig. 3C, lane 1, and Ref. 39). In contrast, induction of ectopic p53 with 100 μM ZnCl₂ for 8.5 h led to rapid whole cell induction of p53 (lane 2). A fraction of the introduced p53 correctly targeted to mitochondria (lane 4), similar to endogenous p53 from ML-1 cells after camptothecin treatment (lane 6). This was followed by the acquisition of apoptosis of EB1 cells, which started at 24 h (10% of cells, 30% at 48 h, and 70% at 72 h) and reached 100% at 96 h, compared with only 4% in uninduced cells.

Third, do mitochondrial regulators of apoptosis influence induction of mitochondrial p53 accumulation? To determine direct functional interrelations at the organellar level, we turned to a cell system that stably overexpresses anti-apoptotic Bcl-2 or Bcl-xL. Parental and vector-transfected mouse 32D myeloid progenitor cells undergo vigorous p53-dependent apoptosis after DNA damage plus IL-3 withdrawal (Fig. 3D, bottom, and Ref. 39). However, cell death, but not cell cycle arrest, is prevented by overexpression of Bcl-2 and Bcl-xL (Fig. 3D, bottom, and Ref. 39). Importantly, Fig. 3D shows that concomitant with rescuing cell viability due to Bcl-mediated mitochondrial stabilization, mitochondrial p53 accumulation, but not total cellular p53 accumulation, is almost completely blocked (compare lanes 6 with 8 and 9). In contrast, vector control 32D cells accumulate mitochondrial p53 after stress (Fig. 3D, compare lanes 2 and 4). Therefore, overexpression of Bcl-2 family members abrogates stress-mediated mitochondrial p53 localization and cell death but not cell cycle arrest. This suggests the existence of a direct or indirect signaling loop between p53 and critical apoptotic regulators like the Bcl-2 family at the mitochondrial level.

**FIG. 4.** Targeting p53 to mitochondria of p53-deficient cells is sufficient to induce apoptosis. A, top row: SaOs-2 cells were transfected with FLAG-tagged wt p53 (wtp53) or (bottom row) mitochondrial import leader fused to FLAG-tagged wt p53 (L-wtp53). Cells were fixed and stained with FLAG antibody to detect p53 (a, c, f, and h) or with mt hsp70 antibody to detect mitochondria (b and g), followed by either fluorescein isothiocyanate-conjugated (a, c, f, and h) or Cy3-conjugated (b and g) secondary antibodies. Nuclei were counterstained by Hoechst (a and f). Brackets indicate confocal analysis after double staining. Images d and i result from superimposing b with c and g with h. Apoptosis was determined by parallel TUNEL assays (e and j); percentages are indicated. Inset, mitochondrial localization of L-wtp53 is verified by cleavage of its leader peptide by mitochondrial endopeptidase. p53 immunoblot (FLAG antibody) of SaOs-2 cells transfected with wt p53 from a (left lane) or with L-wtp53 from f (right lane). B, histogram of a representative experiment (from a total of eight). For each construct, apoptosis (TUNEL positivity) was determined as a percentage of transfected cells (FLAG positivity) by counting 20 random fields each (200–600 cells) (mean ± S.E.; n = 20).
Targeting p53 to Mitochondria Is Sufficient to Induce Apoptosis in p53-deficient Cells—To establish a cause and effect relationship, we targeted wtp53 efficiently into mitochondria of p53 null SaOs-2 cells and compared its apoptotic ability with that of nuclear wtp53. This nuclear bypassing was achieved by generating a FLAG-tagged fusion protein (called L-wtp53) between the N terminus of p53 and the prototypical mitochondrial import leader peptide of human ornithine transcarbamylase (35, 44). Proteins with such a leader signal are recognized at the mitochondrial surface, transported across the double membranes by the multisubunit TOM-TIM complexes, followed by cleavage of the leader in the matrix, and resorting into their final compartments (inner or outer membranes, intermembranous space, or matrix (40, 41). Aside from wtp53, pairs of a truncation mutant (p53, residues 1–305) and a transcriptionally inactive mutant (p53 R175H) were also made. All constructs were compared with a targeted version of the human transcription factor c-REL. All constructs were FLAG-tagged and made in the same expression vector (CMVNeoBam3).

Mitochondrial localization of L-wtp53 in transiently transfected SaOs-2 cells was verified by a distinct mitochondrial FLAG immunofluorescence pattern (Fig. 4A, panel f), co-localization with a mitochondrial marker (mt hsp70) (Fig. 4A, panels g–i), and processing of the fusion protein, indicating in vivo cleavage of the leader peptide by mitochondrial endopeptidase (Fig. 4A, inset). L-wtp53 was undetectable within nuclei (Fig. 4A, panel h), and undetectable levels of nuclear p53 are generally associated with lack of p53 transcriptional activity. In contrast, wtp53 accumulated highly in the nucleus (panel c). Importantly, mitochondrially localized L-wtp53 reproducibly induced apoptosis at least as efficiently as nuclear wtp53 (eight independent experiments, TUNEL assays), indicating that p53 is sufficient to launch an apoptotic pathway from the mitochondrial level. Fig. 4B shows a representative experiment with mitochondrial L-wtp53 inducing 58% apoptosis and nuclear wtp53 inducing 38% apoptosis at 28 h after transfection into SaOs-2 cells. Moreover, a mitochondrially targeted truncated p53 protein, L-p53 (1–305), consistently had the highest apoptotic activity. Again, by immunofluorescence L-p53 (1–305) had a strict mitochondrial distribution pattern and was undetectable within nuclei (data not shown). The latter result suggests that the C terminus including the tetramerization domain is dispensable for mitochondrial p53 function, as opposed to the action of p53 as a transcription factor where the protein is most effective as a tetramer (36, 45–47). In contrast to L-p53 (1–305), nuclear p53 (1–305) was only a poor activator of apoptosis (Fig. 4B), consistent with the poor transactivation abilities of similar p53 truncations (1–290 and 1–320) (48). Finally, to show definitively that the transcriptional activity of p53 is fully dispensable for its mitochondrial action, we tested the effect of a DNA binding mutant (L-p53 R175H) (Fig. 4B). Although its nuclear version was completely inactive as predicted (reviewed in Ref. 1), the targeted protein was equipotent to nuclear wtp53. Importantly, targeting proteins to mitochondria does not kill cells nonspecifically by damaging the organelle, as demonstrated by targeting the transcription factor c-REL with the same leader (L-c-REL) (Fig. 4B). L-c-REL also localized exclusively to mitochondria and expressed at the same level as the targeted p53 proteins (data not shown).

In summary, our data support a direct role of the p53 protein in a novel apoptosis signaling pathway at the mitochondrion, a major transducer of cell death. This pathway is likely to act in synergy with the transcription-dependent mode of action of p53, thereby amplifying its apoptotic potency.

DISCUSSION

We demonstrate here that a fraction of p53 protein is induced to localize to mitochondria in vivo as part of the response in p53-mediated apoptosis. Mitochondrial p53 targeting occurs in a wide spectrum of cell types and after a variety of stress signals including DNA damage and hypoxic stress. Moreover, this event is recapitulated by sole induction of exogenous p53 in p53-null cells (EB1) and precedes the onset of apoptosis in those cells. Mitochondrial localization of p53 was established using a variety of independent approaches including classical cell fractionation, immunoelectron microscopy, and fluorescence-activated cell sorter-based immunodetection. The same approaches were also used for BCL-2 localization to mitochondria (e.g. Refs. 49–52). Mitochondrial p53 localization is specific for p53-dependent apoptosis and does not occur during p53-independent apoptosis, nor does it occur during p53-mediated cell cycle arrest. p53 accumulation to mitochondria is rapid (starting after 1 h) and precedes early dysfunctional changes in mitochondrial membrane potential, cytochrome c release, and procaspase-3 activation. Moreover, mitochondrial anti-apoptotic regulators like BCL-2 and BCL-xL specifically block stress-induced mitochondrial p53 localization and apoptosis but not nuclear p53 induction and cell cycle arrest. This relationship suggests a regulatory feedback signaling loop between p53 and BCL-2 members at the organelar level.

All of the above findings strongly argued that p53 localization to apoptosing mitochondria actively contributes to the apoptotic outcome, rather than being a mere effect thereof. However, although strongly supportive, these findings did not address an active role directly. To show definitively causality, we therefore asked if p53, when deliberately targeted to mitochondria, is competent in inducing apoptosis by itself and without requiring additional DNA damage. In other words, can p53, at least when overexpressed, launch an apoptotic response directly from the mitochondrial level? The answer to this question is yes (Fig. 4). L-wtp53, when redirected from the nucleus and overexpressed in mitochondria, was capable of inducing apoptosis in p53-deficient SaOs-2 cells that was comparable in magnitude to nuclear wtp53. In contrast, a mitochondrially
targeted control transcription factor (L-c-REL) failed to do so. Moreover, even a transcriptionally inactive p53 protein (p53 R175H), at least when expressed at mitochondria at artificially high levels, was as efficient as nuclear wt p53. The latter data rule out any requirement for the transactivation function of p53 when triggering apoptosis from mitochondria. Therefore, excess mitochondrial p53 accumulation, as generated under our experimental conditions, leads to mitochondrial dysfunction that can directly trigger the postmitochondrial caspase cascade and lead to cell death. These data support a novel organellar-based, transcription-independent function of p53. However, under physiologic circumstances, this mitochondrial function is likely an important enhancer to the main pathway, i.e., the transcription-dependent action of p53, in directing apoptosis (Fig. 5). It is important to keep in mind that the levels of p53 that were achieved after deliberate organellar targeting far exceed those that are physiologically induced during stress or are present in mutant p53-expressing cells. This is underlined by SaOs-2 cells transfected with the nuclear p53 175 mutant. The mitochondrial levels of this mutant, which were below immunofluorescence detection (data not shown), were insufficient to rescue the apoptotic response. The same reasoning might be extended to mutant p53-harboring tumor cells that are apoptotically inefficient.

Our data might explain the intriguing observation by Ding et al. (27) who showed that p53 protein from cell-free postnuclear extracts directly activates caspase-3 through an unknown mechanism, provided that extracts were generated from irradiated cells. This result is clear biochemical evidence of an inducible, transcription-independent pro-apoptotic function of p53. Of note, these extracts contained mitochondrial (27) and therefore could have been the source of the p53-dependent effect.

Mitochondrial genome searches of human, mouse, and bovine for the p53 consensus DNA-binding site (RRCWGWYY) did not support the notion that p53 simply acts as a mitochondrial transcription factor. Although we found 2–3 hits, the sites are not near any known transcriptional regulatory sequence and are not highly conserved between species, and therefore likely to be chance occurrences. This negative data are also consistent with the lack of a requirement for mitochondrial DNA for apoptosis (53) and further support the notion of a signaling role of p53 at this site.

The death signal-induced p53 redistribution to mitochondria is somewhat reminiscent of Bax. Bax is a cytosolic protein that undergoes a death signal-induced conformational change (54, 55). This results in rapid relocationalization of Bax to mitochondria which triggers mitochondrial dysfunction and cell death. However, while Bax harbors a transmembrane domain mediating its outer mitochondrial membrane insertion, the precise nature of the mitochondrial association of p53 will require further studies. Our data suggest that p53, after being imported via mt hsp70, distributes to the membranous compartment where it could be a soluble component of the intermembranous space or undergo interaction with another protein on the outer or inner membrane. As in the case of cytochrome c, which lacks the optional amphipathic N-terminal import leader peptide (56), the p53 protein does not have an obvious leader sequence. Concerning the mitochondrial action of p53, it is significant that a truncated targeted version, L-p53(1–305) consistently had the highest apoptotic activity (Fig. 4B), suggesting that the C terminus including the tetramerization domain is dispensable for mitochondrial p53 function. This finding further suggests that the protein domain(s) required in the mitochondrial role of p53 are fundamentally different from the domains required as a transactivator, which depends on C-terminal tetramerization for efficiency. Mapping the mitochondrial effector domain(s) and how they relate to apoptogenic organelar events will elucidate this novel pathway. Li et al. (57) recently showed that p53 signals apoptosis through mitochondria by inducing collapse of MδΨ via reactive oxygen species but not by inducing mitochondrial translocation of Bax or cleavage of Bid.

In summary, we propose a direct p53 signaling pathway that utilizes mitochondria, which are a universal key effector in the apoptotic process of many cell types. This pathway likely acts in synergy with the transcription-dependent mode of action of p53, thereby amplifying the apoptotic potency of p53. Moreover, based on its implication in a broad spectrum of cell types and death signals and the central apoptotic role of mitochondria, this enhancer pathway might be generic for all p53 response genes, whatever the individual set of activated genes in a given cell type/death stimulus might be.
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