Irradiation-induced Translocation of p53 to Mitochondria in the Absence of Apoptosis*

Frank Essmann‡, Stephan Pohlmann‡, Bernhard Gillissen‡, Peter T. Daniel† Klaus Schulze-Osthoff§, and Reiner U. Jänicke††

From the †Institute of Molecular Medicine, University of Düsseldorf, 40225 Düsseldorf, Germany and the ‡Department of Hematology, Oncology and Tumor Immunology, Charite, Humboldt University, 13125 Berlin, Germany

The tumor suppressor protein p53 promotes apoptosis in response to death stimuli by transactivation of target genes and by transcription-independent mechanisms. Recently, it was shown that during apoptosis p53 can specifically translocate to mitochondria, where it physically interacts with and inactivates prosurvival Bcl-2 proteins. In the present study, we therefore investigated the role of mitochondrial translocation of p53 for the stress response of tumor cells. In various cell lines, DNA damage induced by either ionizing irradiation or topoisomerase inhibitors triggered a robust translocation of a fraction of p53 to mitochondria to a similar extent. Nevertheless, the cells succumbed to apoptosis only in response to topoisomerase inhibitors, but remained resistant to apoptosis induced by ionizing radiation. Irradiated cells became senescent, although irradiation triggered a functional p53 response and induced expression of p21, Bax, and Puma. Interestingly, even the targeted expression of p53 to mitochondria was insufficient to activate apoptotic Bcl-2 proteins including Bax, Bak, Bcl-2, Bcl-xL, and others. These Bcl-2 proteins interact with another group of pro-apoptotic proteins that share at least one common motif with Bcl-2, namely the BH3-domain. BH3-only proteins have been proposed to be allosteric regulators of the Bcl-2 proteins and serve as sensors of apoptotic signaling. Upon interaction of anti-apoptotic Bcl-2 proteins with BH3-only proteins, both Bax and Bak undergo a conformational change in response to apoptotic stimuli. This event, which mediates exposure of an occluded N terminus (13, 14), results in the oligomerization of Bax and Bak and in the formation of a mitochondrial outer membrane pore with the subsequent release of cytochrome c. Interestingly, many pro-apoptotic Bcl-2 members, including, for instance, Bax and the BH3-only proteins Puma, Noxa, and Bid, are under the transcriptional control of p53 (15–19).

In addition to the transcriptional role of p53, there is now accumulating evidence for transcription-independent, p53-mediated apoptotic pathways (20–23). In some cell types, for example, p53-dependent apoptosis occurs in the absence of any gene transcription or translation even upon expression of transcriptionally inactive p53 mutants (24–26). The precise molecular mechanisms, however, of how p53 induces apoptosis independently of its transcriptional activity are largely unknown. Intriguingly, p53 has been reported to directly induce mitochondrial cytochrome c release in a Bax-dependent manner in vivo and in vitro, providing evidence for a direct signaling pathway from the p53 protein to the downstream caspase activation cascade (27). These observations were further supported by recent findings demonstrating the appearance of p53 in the cytoplasm or even its translocation to the mitochondria during DNA damage- and stress-induced apoptosis (28–30). Moll and co-workers also found that bypassing the nucleus by targeting p53 expression directly to the mitochondria was sufficient to induce cell death in p53-deficient tumor cells (30). As a mechanistic explanation, it was proposed that p53, like BH3-only proteins, directly induces the permeabilization of the outer mitochondrial membrane by forming inhibitory complexes with anti-apoptotic Bcl-2 and/or Bcl-xL proteins. Green and colleagues (28) have recently shown that the pro-apoptotic Bcl-2 protein Bax is one of the principal cytoplasmic targets of p53 (28). In a comparable scenario, a direct interaction of p53 with mitochondria-localized Bak rather than Bax was proposed that results...
Resistance to Mitochondrial p53-induced Apoptosis

in the disruption of the cyto-protective Mcl-1-Bak complex and hence in the oligomerization and activation of Bak (31). Thus, compelling evidence suggests that p53 directly impacts on mitochondria, although it is still a matter of debate by which route the transcription-independent apoptosis is achieved.

We have previously reported that MCF-7 breast carcinoma cells are sensitive to anti-cancer drug-induced apoptosis, but are remarkably resistant to apoptosis induced by ionizing radiation (IR) regardless of whether or not they express caspase-3 (32). This finding was especially paradox because MCF-7 cells express a transcriptionally active wild-type p53 protein. Thus, we wondered whether the radio-resistant phenotype of these cells might be caused by an improper transcription-independent function of p53. We found that, although IR induced an efficient translocation of p53 to the mitochondria, this process was insufficient to induce apoptosis in MCF-7 and HCT116 carcinoma cells. In addition, in several cell lines that display different apoptosis susceptibilities toward IR or chemotherapeutics, we did not find a correlation between the amount of mitochondrial p53 and apoptosis induction. Interestingly, Bak oligomerization and apoptotic alterations were only observed when cells were transfected with wild-type p53, whereas the expression of p53 targeted directly to the mitochondria did not evoke an apoptotic response. Our results therefore suggest that the resistance to apoptosis induction by mitochondrial p53 might contribute to the radio-resistant phenotype of tumor cells.

MATERIALS AND METHODS

Cells, Reagents, and Antibodies—MCF-7 breast carcinoma cells and its subclones MCF-7/casp-3 (33), MCF-7 cells stably expressing a GFP-Bax fusion protein, as well as MCF-7/p53siRNA (34) and MCF-7/casp-3/p53siRNA cells (35), were cultured in RPMI 1640. The human B-lymphoblastoid cell line SKW6.4 and RKO colon carcinoma cells were cultured in Iscove's modified Dulbecco's medium and Dulbecco's modified Eagle's medium, respectively. All media were supplemented with 10% heat-inactivated fetal calf serum, 10 mM glutamine, and 100 μg/ml of each streptomycin and 100 units/ml penicillin (PAA Laboratories GmbH, Co¨lbe, Germany). The HCT116 and HCT116/p53−/− cells were maintained in McCoy’s 5A medium supplemented as above. The monoclonal Chk1 antibody was obtained from Transduction Laboratories (Heidelberg, Germany), and the monoclonal p53 antibody (Ab-6) from Calbiochem (Bad Soden, Germany). The p21 and poly(ADP-ribose) polymerase monoclonal antibodies were from BD Pharmingen, and the polyclonal antibody to high mobility group 1 (HMGI) and the monoclonal antibody to Tom20 from BD Bioscience. The monoclonal antibodies to Bak and Bcl-2 were purchased from Oncogene Research Products (San Diego, CA) and Novocastra Laboratories (Newcastle, UK), respectively. Bcl-xL and Bax antibodies were from Trevigen (Gaithersburg, MD), the conformation-specific anti-Bax-NT antibody was from Upstate Biotechnology, The polyclonal rabbit antibody recognizing Puma as well as the monoclonal antibody against proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz (Heidelberg, Germany) and the antibodies directed against caspase-3 and actin were from R&D Systems (Wiesbaden, Germany) and Sigma, respectively. Secondary antibodies for the immunofluorescence studies including chicken anti-mouse and anti-rabbit antibodies coupled to Alexa Fluor 488 and Alexa Fluor 594, respectively, or goat anti-mouse antibody coupled to Alexa Fluor 350 as well as Mitotracker Red were from Molecular Probes (Leiden, The Netherlands). Peroxidase-labeled antibodies to rabbit and mouse IgG were from Promega GmbH (Mannheim, Germany). The nuclear stain 4′,6-diamidino-2-phenylindole was from Sigma (Deisenhofen, Germany). For protease inhibition, buffers were supplemented with a protease inhibitor mixture (Roche Diagnostics).

Treatment and Transfection of Cells—Cells were exposed to ionizing radiation at the indicated dose using a Gammacell 1000 Elite (Nordion International Inc., Fleurus, Belgium) in the presence or absence of caffeine (1 mM, Sigma). Alternatively, cells were treated with the topoisomerase inhibitors etoposide (10 μM) or camptothecin (5 μM; both Sigma). The term “radio-resistant” that is used throughout the entire manuscript refers to “resistant to IR-induced apoptosis.” Transfections of MCF-7 cells were performed with FuGENE reagent according to the instructions of the manufacturer (Roche Diagnostics). Mammalian expression vectors encoding for wild-type p53 (p53wt) or a p53 fused to the transmembrane domain of Bcl-2 (p53-CTM) were published before (30) and kindly provided by U. Moll. Stably transfected MCF-7 cells expressing a GFP-tagged Bax construct (36) were selected in G418 (1 mg/ml).

Immunofluorescence Microscopy—For immunofluorescence staining, cells were seeded on coverslides. At the indicated time points, cells were fixed for 20 min in ice-cold PBS containing 2% formaldehyde. Blocking and all following procedures were performed in PBS supplemented with 4% BSA and 0.05% saponin. Primary antibodies were applied at 4 °C overnight at the appropriate concentration. Then, cells were washed three times for 20 min and incubated with the secondary antibody (1:500). Finally, samples were washed extensively and mounted in fluorescent mounting medium (Dako Corporation, Carpinetra, CA) with or without 10 ng/ml 4′,6-diamidino-2-phenylindole. Pictures were taken on an Axiovert 135 Microscope (Zeiss, Germany) with an Apochromat ×63 oil immersion lens using OpenLab software (Improvision, Tübingen, Germany).

Flow Cytometric Analysis—For cell cycle analysis and detection of apoptosis, samples were prepared according to Nicolletti et al. (37). Briefly, cells were harvested by trypsinization, washed in cold PBS, and resuspended in lysis buffer containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml propidium iodide. All flow cytometric analyses were performed on a FACScalibur (BD Biosciences) by using CellQuest analysis software. For each determination a minimum of 10,000 cells was analyzed.

Senescence-associated β-Galactosidase Staining—Staining for β-galactosidase activity was performed as previously described (38). Briefly, after the indicated treatment cells were washed with PBS, fixed in 0.2% glutaraldehyde and 2% formaldehyde for 5 min, washed again with PBS, and finally stained in the absence of CO2 in staining solution (150 mM NaCl, 2 mM MgCl2, 5 mM K3Fe(CN)6, 5 mM K2Fe(CN)6, 40 mM citric acid, and 12 mM sodium phosphate, pH 6.0) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

Cell Extracts and Subcellular Fractionation—Total cell extracts were prepared in high-salt lysis buffer containing 1% Nonidet P-40, 20 mM HEPES (pH 7.9), 2 mM phenylmethylsulfonyl fluoride, 350 mM NaCl, 20% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol and protease inhibitors. For isolation of mitochondria, cells were harvested by scraping, centrifuged, washed, and resuspended in ice-cold buffer MB (400 mM sucrose, 50 mM Tris/HCl, pH 7.6, 1 mM EGTA, 5 mM 2-mercaptoethanol, 10 mM potassium phosphate, pH 7.6, and 0.2% BSA). After incubation on ice for 20 min, cells were Dounce homogenized with 40 strokes, and unbroken cells and nuclei were
removed by centrifugation at 4,000 × g for 1 min. The supernatant was centrifuged for 10 min at 15,000 × g to pellet mitochondria. The resulting supernatant was removed and designated the cytoplasmic fraction, whereas the pellet enriched for mitochondria was resuspended in buffer MSM (300 mM mannitol, 10 mM potassium phosphate, pH 7.2, 0.1% BSA) and was referred to as the M1 fraction. To obtain highly purified mitochondria, the M1 fraction was loaded onto a discontinuous 1.2–1.6 M sucrose gradient buffered with 10 mM Tris/HCl (pH 7.5) and 0.1% BSA. After centrifugation at 70,000 × g for 1 h, the interphase was harvested and diluted in MSM buffer at a ratio of 1:4. Mitochondria were centrifuged at 45,000 × g for 15 min, and the resulting pellet was designated M2.

**Western Blot Analysis**—Protein concentrations were determined using the BCA assay kit (Pierce Biotechnology), and 15 μg of protein per lane were loaded onto standard SDS-PAGE gels and separated at 200 V. The proteins were transferred onto a 0.2-μm pore size polyvinylidene difluoride membrane (Amersham Bioscience) by semi-dry blotting at a constant current of 40 mA for 90 min in a buffer containing 10 mM CAPS (pH 11) and 10% MeOH. The membranes were blocked in PBS containing 4% BSA and 0.05% Tween 20 for 1 h, followed by an overnight incubation with the primary antibody at 4 °C. After washing the membranes extensively, the appropriate secondary antibodies (1:5000) were applied for 1 h. Membranes were washed for an additional 2 h in PBS, 0.05% Tween 20, and proteins were visualized using ECL reagents (Amersham Biosciences).

**RESULTS**

**Irradiation Induces a Functional p53 Response in the Absence of Apoptosis**—The tumor suppressor p53 induces apoptosis in response to DNA damage in diverse cellular systems. Nevertheless, MCF-7 breast carcinoma cells are resistant to apoptosis induced by IR regardless of whether or not they express caspase-3, although they harbor a functional p53 gene (32, 35). Hence, we wondered whether the resistance of MCF-7 and MCF-7/casp-3 cells to IR-induced apoptosis could be caused by a mechanism rendering wild-type p53 unresponsive toward IR in these cells. However, exposure to IR induced significant p21 expression in both MCF-7 cell lines, a process that is most likely mediated via stabilization and transcriptional activation of p53 (Fig. 1A). In addition, irradiated MCF-7 and MCF-7/casp-3 cells displayed a persistent G2/M arrest (Fig. 1B) that correlated with the induction of cellular senescence as evidenced by staining of the senescence-associated β-galactosidase activity and a dramatic increase in the cell volume (Fig. 1C). IR-induced senescence was substantially reduced in MCF-7 and MCF-7/casp-3 cells stably transfected with a p53iRNA construct (35), implying...
Resistance to Mitochondrial p53-induced Apoptosis

Mitochondrial Translocation of p53 in Response to Irradiation—Accumulating evidence suggests that p53 can also induce apoptosis via transcription-independent events that are only poorly understood. One of these events might be the DNA damage-induced translocation of p53 to the mitochondria that was previously described (29, 30). To examine whether this was a critical event for the apoptosis resistance of irradiated MCF-7 cells, we initially analyzed the subcellular localization of p53 by immunofluorescence microscopy. Compared with untreated cells, irradiation triggered an increase in nuclear size associated with the formation of micronuclei (Fig. 2A). Unlike expression of the nuclear HMG1 that remained unaffected by IR, p53 expression was barely detectable in untreated MCF-7 cells, but was highly induced in irradiated cells (Fig. 2A). However, IR-induced p53 was predominantly found in the nucleus, and no mitochondrial-associated p53 could be detected with this technique. Also the use of different antibodies recognizing either N- or C-terminal p53 epitopes did not result in the detection of endogenous mitochondria-associated p53 following IR (data not shown). Although this result would support our initial hypothesis that the radio-resistant phenotype of MCF-7 cells might be caused by an improper p53 function, we had to rule out the possibility that the M1 fractions contained also nuclear or cytoplasmic p53 that was not specifically translocated to mitochondria during IR treatment in vivo, but was simply co-sedimented during the purification procedure. Western blot analyses revealed that the mitochondrial M1 fractions of both cell lines were indeed contaminated with nuclear proteins such as the PCNA but, for unknown reasons, not with poly(ADP-ribose) polymerase (Fig. 2B). Thus, the observed p53 signals in the M1 fractions of irradiated cells might only reflect contaminations with nuclear p53. To avoid such nuclear contaminations, and to undoubtedly establish whether IR induces the mitochondrial translocation of p53, we further purified the mitochondria-enriched M1 fractions by discontinuous sucrose gradient centrifugation. Western blot analyses of samples obtained with such a purification procedure (M2 fractions) now clearly revealed an irradiation-induced translocation of p53 to the mitochondria in both MCF-7 lines (Fig. 2B). Only the mitochondrial marker proteins Tom20 and cytochrome c (data not shown), but not the nuclear poly(ADP-ribose) polymerase or PCNA proteins were detected together with p53 in the M2 fraction of irradiated cells, confirming the composition and purity of these samples. In addition, these data suggest that the mitochondrial p53 translocation must have occurred in vivo as a response of the cell to IR exposure, but not because of an in vitro co-sedimentation artifact caused by the preparation procedure.
It was reported previously that a fraction of p53 only localizes to mitochondria under conditions that provoke p53-dependent apoptosis, but not in apoptosis-resistant cells (28, 30). However, we now show that IR induces also the mitochondrial translocation of p53 in MCF-7 and MCF-7/casp-3 cells that are highly resistant to apoptosis induced by IR. In an attempt to explain this discrepancy, we prepared sucrose gradient fractions from MCF-7/casp-3 cells that were exposed to IR in combination with the methylxanthine and ATM inhibitor caffeine, a treatment that efficiently induces apoptosis in these cells (35). When compared with purified M2 fractions of cells that were only irradiated, co-treatment with caffeine resulted in even further increased p53 levels at the mitochondria (Fig. 2C). At first glance, this result would therefore support the idea that mitochondrial translocation of p53 correlates with apoptosis induction. However, IR-induced apoptosis in the presence of caffeine proceeds in a p53-independent manner in MCF-7/casp-3 cells (35).

Mitochondrial p53 Translocation in HCT116 Cells—In light of the surprising finding that IR induces the mitochondrial translocation of p53 in MCF-7 and MCF-7/casp-3 cells that are both resistant to irradiation-induced apoptosis, we extended our investigation to another experimental system. As demonstrated by FACS analyses, HCT116 colon carcinoma cells were also resistant to apoptosis induced by high dose IR regardless of whether or not they express p53 (Fig. 3A). Similar to MCF-7 cells (32), HCT116 cells displayed a persistent irradiation-induced G2/M arrest, but were efficiently killed by the anti-cancer drug etoposide in a p53-dependent manner verifying their p53 status (Fig. 3A). Western blot analyses confirmed these observations and demonstrated the disappearance (and hence, activation) of the pro-caspase-3 protein predominantly in etoposide-treated HCT116 cells expressing wt-p53 (Fig. 3B, lower panel). Expression of p21 was clearly dependent on p53, as it was not observed in p53-deficient HCT116 cells (Fig. 3B). More importantly, p53 was found together with the mitochondrial marker protein Tom20 in the highly purified M2 fractions of both irradiated and etoposide-treated wild-type HCT116 cells, regardless of whether or not the cells were killed by this treatment (Fig. 3C). Again, the mitochondrial accumulation was specific for p53 because other nuclear proteins such as PCNA (Fig. 3C) and HMG1 (data not shown) were not detected in the M2 fraction. Thus, the specific mitochondrial
translocation of p53 in the absence of apoptosis is not only restricted to the MCF-7 cell system, but occurs also in HCT116 colon carcinoma cells that are largely resistant to IR-induced apoptosis.

Mitochondrial Translocation of p53 Does Not Correlate with Apoptosis Susceptibility—It was recently reported that mitochondrial translocation of p53 occurs specifically during p53-dependent apoptosis, but not during p53-mediated cell cycle arrest or in p53-independent apoptosis (20). Our data, however, demonstrate p53 accumulation at the mitochondria following a treatment (IR) that does not induce apoptosis in two different cell lines. To investigate this discrepancy in more detail, we compared apoptosis susceptibility and mitochondrial p53 accumulation also in the RKO colon carcinoma cell line. In agreement with a previous report (29), we found that these two events correlated well when the cells were treated for 6 h with camptothecin, which efficiently induced apoptosis (Fig. 4A) and p53 accumulation at the mitochondria (Fig. 4B). We detected, however, comparable amounts of mitochondrial p53 when the cells were exposed to IR, a treatment that does not induce apoptosis in these cells (Fig. 4). Similar results were obtained when the effects of both treatments were compared in HCT116 and MCF-7/casp-3 cells (Fig. 4). In addition, we did not observe a correlation with regard to apoptosis sensitivity and mitochondrial p53 accumulation when we performed the experiments with the human B-lymphoblastoid cell line SKW6.4. Although these cells were equally susceptible to apoptosis induction by both treatments, a significantly larger portion of p53 accumulated at the mitochondria following incubation with camptothecin (Fig. 4). Interestingly, neither cell death nor p53 translocation appears to correlate with expression of the anti-apoptotic Bcl-2 protein at the mitochondria. Whereas the radio-resistant cell lines HCT116, MCF-7/casp-3, and RKO express extremely low and moderate levels of Bcl-2, respectively, we found very high Bcl-2 expression in IR-sensitive SKW6.4 cells (Fig. 4B).

Bax and Puma Expression Do Not Correlate with Apoptosis Susceptibility—Although we found in several cell lines no correlation between apoptosis susceptibility and mitochondrial p53 accumulation, we wondered whether the resistant phenotype of these cells to IR-induced apoptosis might be caused by defects in events downstream of mitochondrial p53. To address this possibility, we analyzed in MCF-7 cells expression of the p53-inducible pro-apoptotic proteins Bax and Puma that play key roles in apoptotic pathways mediated by mitochondrial p53 (17, 23). Western blot analyses of whole cell extracts of MCF-7/casp-3 and MCF-7/casp-3/p53siRNA cells showed that IR clearly induced up-regulation of Bax and Puma in a p53-dependent manner, whereas expression of other Bcl-2 family members such as Bak, Bcl-2, or Bcl-xL was neither affected by this treatment nor by the p53 status of the cells (Fig. 5A). Interestingly, when we analyzed the mitochondrial M2 fractions of several cell lines exposed to either IR or the chemotherapeutic drugs etoposide or camptothecin, we did not observe a correlation between Puma expression and apoptosis susceptibility. Puma was induced in all cell lines by either treatment regardless of whether or not the cells succumbed to apoptosis (Fig. 4B). In fact, at both time points analyzed (6 h and 24 h), IR induced an even higher Puma expression in the mitochondrial M2 fraction of IR-resistant HCT116 cells than etoposide or in IR-sensitive SKW6.4 cells. Furthermore, we found similar Puma levels at the mitochondria of MCF-7/casp-3 and RKO cells exposed to either IR or to chemotherapeutic drugs although only the latter treatment induces efficient apoptosis in these cells. Thus, neither the mitochondrial translocation of p53 nor expression of Bcl-2 or Puma appear to correlate with apoptosis sensitivity in the cell lines investigated. Although these data do not rule out a possible contribution of these and other Bcl-2 proteins toward the apoptosis-resistant phenotype of the cell lines investigated, the observation that Bax and Puma can be induced by IR in these cells indicates the functionality of the p53 pathway.

Bax Activation in MCF-7 Cells—Next, we tested the functionality of Bax that is known to be located upstream of caspase activation in the apoptosis signaling pathway. Bax exerts pro-apoptotic activity upon its conformational change and the exposure of an occluded N terminus, resulting in Bax oligomerization and cytochrome c release. Using an antibody that specifically detects the N terminus of such a conformationally changed, i.e. active, Bax protein, we could demonstrate activation of Bax only in etoposide-treated MCF-7 cells, but not in cells that were exposed to IR (Fig. 5C). Analysis of an overexpressed GFP-Bax
Resistance to Mitochondrial p53-induced Apoptosis

FIGURE 5. Bax expression and activation only in etoposide-treated, but not in irradiated MCF-7 cells. A, Western blot analyses for the status of Bax and other Bcl-2 and Bcl-XL-only proteins in total extracts of untreated and irradiated (20 gray) MCF-7/casp-3 and MCF-7/casp-3/p53siRNA cells 24 and 48 h post-IR. The asterisk indicates an unspecific band. B, Western blot analysis for the status of Puma in the cytosol (Sup) and in mitochondrial M2 fractions of the indicated cell lines that were either left untreated (control) or analyzed after the indicated times following exposure to 20 gray (IR). 10 μM etoposide, or 5 μM camptothecin. C, MCF-7 (left panel) and MCF-7/GFP-Bax cells (right panels) were analyzed 24 h post-irradiation (20 gray) by immunofluorescence with either an antibody specific for conformationally changed (activated) Bax (Bax-NT) (lower panels), or by 4′,6-diamidino-2-phenylindole staining (upper left panel) and GFP expression (upper right panel). No clustering of Bax could be detected in irradiated cells (left and middle panels). Etoposide treatment (24 h), however, induced Bax oligomerization as indicated by the dotted GFP-Bax expression pattern (upper right panel), which is congruent with the Bax-NT stained cells (lower right panel).

FIGURE 6. Expression of wild-type p53 but not p53 targeted to mitochondria induces Bax clustering and apoptosis in MCF-7 cells. MCF-7 cells transfected with p53-wt (B, lower panel) or with a mitochondria-targeted p53 construct (p53-CTM) (A and B, upper panel) were analyzed 36 h post-transfection by immunofluorescence for p53 expression and for Bax activation with the Bax-NT antibody. Mitochondrial expression of p53-CTM (A) was confirmed by staining the cells also with Mitotracker red. Transfection of MCF-7 cells with p53-wt (B, lower panel), but not with p53-CTM (B, upper panel) resulted in the appearance of double positive-stained cells indicating Bax oligomerization in p53-overexpressing cells. Note that the two cells stained positive for active Bax (B, upper panel) do not express the p53-CTM construct and hence represent apoptotic cells that have died spontaneously. The experiments were repeated at least four times yielding similar results. C, Western blot analysis (lower panel) of p53 expression in MCF-7 cells that were either untransfected (c), transfected with p53-wt (wt) or with the mitochondria-targeted p53 construct (CTM). Expression of an unrelated band serves as loading control. Pro-apoptotic effects were quantitated by counting cells that stained double positive for p53 and active Bax (Bax-NT) in 20 random microscopic fields (upper panel).
Resistance to Mitochondrial p53-induced Apoptosis

The possibility that the different apoptotic outcome upon transfection of these two constructs is caused by their differential expression, our results provide strong evidence that neither endogenous p53 upon IR treatment nor transiently expressed p53 targeted to mitochondria are able to induce Bax oligomerization and subsequent apoptotic events in MCF-7 cells.

DISCUSSION

For the last few years, substantial evidence has emerged suggesting the existence of p53-dependent apoptosis pathways that do not require its transcriptional activity. Recently, p53 was demonstrated to translocate into the cytoplasm and even onto mitochondria during DNA damage- and stress-induced apoptosis (28–30). Although it is still controversially discussed by which mechanism and interaction partners, in particular of the Bcl-2 family, p53 exerts an extranuclear, pro-apoptotic function, p53 may directly impact on mitochondria. p53 was shown to interact either with anti-apoptotic Bcl-2 proteins leading to the inhibition of their cytoprotective function (30), whereas in other studies a direct or indirect interaction with the pro-apoptotic Bcl-2 members Bak or Bax has been proposed (28, 31). A mitochondrial translocation of p53 was observed in a variety of human and mouse cell lines and occurred specifically only in p53-dependent apoptosis such as in drug-, hypoxia-, or radiation-induced apoptosis, but was not detected during p53-mediated cell cycle arrest or in p53-independent apoptosis (20). In addition, mitochondrial p53 appears to contribute also to the physiological p53 stress response in vivo, as it could be detected following IR or intravenous application of etoposide in several radio-sensitive murine organs including thymus, spleen, testis, and brain, but not in radio-resistant organs such as liver and kidney (40).

Based on these and other reports it was surprising to find that IR treatment induced the translocation of p53 to mitochondria also in MCF-7 and MCF-7/casp-3 cells that display a persistent G1/M arrest following IR, but are resistant to apoptosis induction by this treatment (32). Although the IR-induced mitochondrial p53 levels were below the immunofluorescence detection limit, the presence of p53 at the mitochondria following IR could be readily demonstrated by Western blot analyses of subcellular fractions (M1 and M2). Only the M1 fractions were partially contaminated with nuclear proteins, whereas composition and purity of the M2 fractions could be confirmed by the absence and presence of nuclear and mitochondrial marker proteins such as HMG1, PCNA, and Tom20, respectively. Irradiation-induced translocation of p53 to mitochondria without any signs of apoptosis was not restricted to the MCF-7 cell line, but was also a prominent feature of similarly treated HCT116 and RKO cells. In addition, we did not observe a correlation between mitochondrial p53 and the extent of apoptosis as exposure of various cell lines to IR, etoposide, or camptothecin resulted in comparable levels of mitochondrial p53, whereas their apoptotic responses toward these stimuli differed dramatically. Hence, our data that are in line with a very recent report showing that a transcriptional inactive p53 mutant is completely unable to induce apoptosis in vitro and in vivo (41), demonstrate that the physical translocation of p53 to mitochondria is not necessarily associated with the induction of apoptosis as it was proposed previously (29, 30).

Although the exact reason for this discrepancy is presently unknown, one possible explanation might be that too little amounts of p53 protein are translocated to the mitochondria following IR treatment. Provided that the apoptotic machinery is only activated above a certain threshold level of mitochondrial p53, the IR-induced p53 levels at the mitochondria might simply be not sufficient to induce apoptosis. Several observations, however, argue against this explanation and clearly suggest that even elevated mitochondrial p53 levels do not trigger an apoptotic response at least in MCF-7 cells.

First, as quantitated by densitometry of the immunoblots, mitochondrial p53 comprised ~2% of the total induced p53 in MCF-7 or MCF-7/casp-3 cells. This amount is very similar to the mitochondrial fraction of p53 in other cell types in which mitochondrial p53 was originally proposed to provoke apoptosis (29). Second, although we found even further increased p53 levels associated with mitochondria in M2 fractions of MCF-7/casp-3 cells that were exposed to IR in the presence of caffeine than compared with cells exposed to IR alone, apoptosis induced by the combined treatment of IR and caffeine proceeds in a p53-independent manner (35). Hence, these results suggest that elevation of mitochondrial p53 levels induced by this treatment does not contribute to the rate of apoptosis in these cells. In addition, these data demonstrate that mitochondrial translocation of p53 can be also observed in p53-independent apoptosis systems, a finding that is in sharp contrast to previous studies (30, 40). Third, it is well accepted that the ratio of pro- and anti-apoptotic Bcl-2 proteins is an important factor determining the fate of a DNA-damaged cell. As p53 was shown to interact either with the pro-apoptotic Bcl-2 proteins Bax or Bak (28, 31) or with the anti-apoptotic proteins Bcl-2 and Bcl-xL, it can be assumed that aberrant expression of either of these proteins would interfere with the pro-apoptotic activity of mitochondrial p53. However, MCF-7 cells do not express abnormal levels of either Bcl-2 or Bcl-xL, and Bax or Bak, respectively. In fact, IR treatment of MCF-7/casp-3 cells solely increased expression of the pro-apoptotic proteins Bax and Puma without affecting Bak, Bcl-2, or Bcl-xL levels. Hence, also these results do not favor the above mentioned threshold hypothesis. This view is also supported by our findings that Bcl-2 and Puma levels neither correlate with apoptosis sensitivity nor with the amount of p53 translocated to the mitochondria following exposure of the cells to IR, camptothecin, or etoposide.

Finally, and most importantly, we have shown that even MCF-7 cells that are deficient for caspase-3 displayed oligomerized Bax (Fig. 6) and Bak (data not shown) and succumbed to apoptosis following overexpression of a wt-p53 construct. In contrast, when MCF-7 cells were transfected with a mitochondria-targeted expression construct of wt-p53 (p53-CTM) that was previously postulated to efficiently induce apoptosis (30), no activation of Bax or Bak (data not shown) or any other apoptotic sign was observed in these cells. Therefore, our data demonstrate that not only the IR-induced mitochondrial translocation of endogenous p53, but even the targeted overexpression of exogenous mitochondrial p53 is insufficient to induce apoptosis of MCF-7 cells. As chemotherapeutic drugs or overexpression of wt-p53 were sufficient to induce Bax activation and apoptosis, our data suggest that the radio-resistant phenotype of these cells is not caused by a general resistance or unresponsiveness to p53-dependent cell death. Nevertheless, one has to keep in mind that apoptosis induction by overexpression of wt-p53 is most likely achieved via its transcriptional activity as overexpressed wt-p53 was mainly located in the nucleus, but was never detected at the mitochondria.

It is still possible that the inability of mitochondrial p53 to induce Bax oligomerization and apoptosis is caused by the absence or presence of certain inhibitory or stimulatory factors. In addition, it might well be the case that the interaction of mitochondrial p53 with Bcl-2 or Bcl-xL is for unknown reasons hampered in these cells, a scenario that would easily explain their unresponsiveness toward mitochondrial p53. Also the lack of post-translational modifications of p53 itself that might be required for its pro-apoptotic activity at mitochondria might account for the observed resistance. With regard to phosphorylation and acetylation,
Acknowledgments—We thank Drs. U. Moll and B. Vogelstein for their kind gift of plasmids and cells.

REFERENCES

1. Vogelstein, B., Lane, D., and Levine, A. J. (2000) Nature 408, 307–310
2. Vousden, K. H., and Lu, X. (2002) Nat. Rev. Cancer 2, 594–604
3. Bargonetti, J., and Manfredi, J. J. (2002) Curr. Opin. Oncol. 14, 86–91
4. Oren, M. (2003) Cell Death Differ. 10, 431–442
5. Vousden, K. H., and Lu, X. (2002) Cancer Res. 62, 49–53
6. Vousden, K. H., and Prives, C. (2005) Cell 120, 7–10
7. Olivier, M., Eedes, R., Hollstein, M., Khan, M. A., Harris, C. C., and Hainaut, P. (2002) Hum. Mutat. 19, 607–614
8. Owen-Schaub, L. B., Zhang, W., Cusack, J. C., Angelo, L. S., Santee, S. M., Fujiwara, T., Vousden, K. H., and Prives, C. (2005) Mol. Cell. Biol. 25, 153–164
9. Wu, G. S., Burns, T., McDonald, E. R., 3rd, Jiang, W., Meng, R., Krantz, I. D., Kao, G., Gan, D. D., Zhou, J. Y., Muschel, R., Hamilton, S. R., Spinner, N. B., Markowitz, S., Wu, G., and El-Deiry, W. S. (1997) Nat. Genet. 17, 141–143
10. Bennett, M., Macdonald, K., Chan, S. W., Luzio, J. P., Simari, R., and Weissberg, P. (1998) Science 282, 290–293
11. Soengas, M. S., Alarcon, R. M., Yoshiada, H., Giaccia, A. J., Hakem, R., Mak, T. W., and Lowe, S. W. (1999) Science 284, 156–159
12. Johnstone, R. W., Rueff, A. A., and Lowe, S. W. (2002) Cell 108, 153–164
13. Nychushin, A., Smith, C. L., Hsu, Y. T., and Youle, R. J. (1999) EMBO J. 18, 2330–2341
14. Desagher, S., Osten-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. C. (1999) J. Biol. Chem. 274, 8910–8916
15. Miyashita, T., Kitada, S., Krajewski, S., Horne, W. A., Delia, D., and Reed, J. C. (1995) J. Biol. Chem. 270, 26049–26052
16. Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000) Science 288, 1053–1058
17. Nakano, K., and Vousden, K. H. (2001) Mol. Cell 7, 683–694
18. Kannan, K., Amariglio, N., Rechavi, G., Jakob-Hirsch, J., Kela, I., Kaminski, N., Getz, G., Domany, E., and Givol, D. (2001) Oncogene 20, 2225–2234
19. Sax, J. K., and El-Deiry, W. S. (2003) Cell Death Differ. 10, 413–417
20. Moll, U. M., and Zaika, A. (2001) FEBS Lett. 493, 65–69
21. Chipuk, J. E., and Green, D. R. (2003) J. Clin. Investig. 111, 995–1004
22. Erster, S., and Moll, U. M. (2005) Biochem. Physiol. Biocommun. 331, 843–850
23. Yee, K. S., and Vousden, K. H. (2005) Carcinogenesis 26, 1317–1322
24. Caelles, C., Helbing, A., and Karin, M. (1994) Nature 370, 220–223
25. Haupt, Y., Rowan, S., Shaulian, E., Kazaz, A., Vousden, K., and Oren, M. (1997) Leukemia 11, Suppl. 3, 337–339
26. Haupt, Y., Rowan, S., Shaulian, E., Vousden, K. H., and Oren, M. (1995) Genes Dev. 9, 2170–2183
27. Schuler, M., Bossy-Wetzel, E., Goldstein, J. C., Fitzgerald, P., and Green, D. R. (2000) J. Biol. Chem. 275, 7337–7342
28. Chipuk, J. E., Kowarski, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., and Green, D. R. (2004) Science 303, 1010–1014
29. Marchenko, N. D., Zaika, A., and Moll, U. M. (2000) J. Biol. Chem. 275, 16202–16212
30. Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003) Mol. Cell 11, 577–590
31. Leu, J., Dumont, P., Hafey, M., Murphy, M. E., and George, D. L. (2004) Nat. Cell Biol. 6, 443–450
32. Janicke, R. U., Engels, I. H., Dunkern, T., Kaina, B., Schulze-Osthoff, K., and Porter, A. G. (2001) Oncogene 20, 5043–5053
33. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (2001) J. Biol. Chem. 276, 15608–15614
34. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) Science 296, 550–553
35. Essmann, F., Engels, I. H., Totzek, G., Schulze-Osthoff, K., and Janicke, R. U. (2004) Cancer Res. 64, 7065–7072
36. von Haeften, C., Gillissen, B., Hemmati, P. G., Wendid, J., Guner, D., Mrozek, A., Belka, C., Durken, B., and Daniel, P. T. (2004) Oncogene 23, 8320–8332
37. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991) J. Cell Biol. 113, 65–69
38. Schuler, M., Bossy-Wetzel, E., Goldstein, J. C., Fitzgerald, P., and Green, D. R. (2004) J. Cell Biol. 162, 1053–1064
39. Dimri, G. P., Lee, J. M., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linkens, K. M., Rubelj, I., Pereira-Smith, O., Peacocke, M., and Campisi, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9363–9367
40. von Haeften, C., Gillissen, B., Hemmati, P. G., Wendid, J., Guner, D., Mrozek, A., Belka, C., Durken, B., and Daniel, P. T. (2004) Oncogene 23, 8320–8332
41. Nister, M., Tang, M., Zhang, X.-Q., Yin, C., Beeche, M., Hu, X., Enblad, G., van Dyke, T., and Wahl, G. M. (2005) Oncogene 24, 3536–3573

Resistance to Mitochondrial p53-induced Apoptosis

no major differences were observed in the modification patterns between the pro-apoptotic mitochondrial and nuclear p53 proteins (39). However, these data do not rule out that other post-translational events, such as ubiquitinylation or sumoylation, might play a role in apoptosis induction by mitochondrial p53. Finally, we cannot exclude the possibility that translocation of p53 to mitochondria is merely a consequence of DNA damage signaling without any implications for the fate of the cell.

In summary, we show that the IR-induced translocation of p53 to the mitochondria is not unequivocally associated with apoptosis and that mechanisms might exist that counteract the pro-apoptotic ability of mitochondrial p53. This could be convincingly demonstrated in several cell lines of different origin. The reason why most of these cell lines are resistant to apoptosis induction by mitochondrial p53 is completely unknown, but is especially intriguing as it might represent a potential mechanism that contributes to the occurrence of a radio-resistant tumor cell phenotype.