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Essential Role for Caspase-8 in Transcription-independent Apoptosis Triggered by p53*

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p53’s dual regulation of arrest versus apoptosis may underlie tumor-selective effects of anti-cancer therapy. p53’s apoptotic effect has been suggested to involve both transcription-dependent and -independent mechanisms. It is shown here that caspase-8 is activated early in cells undergoing p53-mediated apoptosis and in S100 cell-free extracts that recapitulate transcription-independent apoptosis. Depletion or inactivation of caspase-8 either in cells or cell-free extracts completely prevents this transcription-independent apoptosis and significantly attenuates overall death induced by wild-type p53. Importantly, caspase-8 activation appears to be independent of FADD, and caspase-8 is found in a novel 600-kDa complex following p53 activation. These findings highlight the roles of both transcription-dependent and -independent apoptosis by p53 and identify an essential role for caspase-8 in the transcription-independent pathway.

Dysregulation of apoptosis plays a major role in the development of cancer (1). p53 modulates the apoptotic response in tumor cells following signals such as DNA damage, growth factor deprivation, and hypoxia (2–4). p53-mediated apoptosis has been implicated as an important mechanism by which many antitumor treatments kill cancer cells (5, 6). Its unique ability to trigger cell cycle arrest (7) or apoptosis (8–10) suggests that p53 may confer a favorable therapeutic index to cancer cells during antineoplastic treatment (11, 12). The mechanism(s) by which p53 modulates activation of caspases during apoptosis remain incompletely understood. p53 is a sequence-specific transcription factor (2–4). A number of studies provide evidence that transcriptional activation by p53 is required for apoptosis in some experimental systems. For example, baby rat kidney cells stably expressing E1A and a temperature-sensitive p53 mutant underwent apoptosis when shifted to the permissive temperature, whereas baby rat kidney cells transformed by E1A and a transcriptionally defective temperature-sensitive p53 mutant failed to do so (13). In another study, microinjection of wild-type p53, but not transcription-defective mutants, into p53-null mouse embryo fibroblasts (MEFs)1 expressing E1A induced apoptosis. Moreover, a chimeric transcription activator protein in which the p53 transcription activation domain located at the amino-terminal region was replaced with that of herpes simplex virus VP16 protein remained competent in mediating apoptosis, arguing that transcription activation, rather than the amino-terminal region of p53 per se, is important for the p53 apoptotic function (14). Consistent with these findings, several transcriptional target genes of p53 have been identified whose up-regulation has been shown to promote apoptosis (15–18).

By contrast, there is also strong evidence that p53 can induce apoptosis independent of its transcription function. Using three somatotopic cell lines that express a temperature-sensitive p53 mutant, Caeles et al. (19) found that a shift to the permissive temperature (32.5 °C) triggers apoptosis following UV-C irradiation even in the presence of transcriptional and translational inhibitors. Similarly, activation of Myc in MEFs expressing a temperature-sensitive p53 mutant induced apoptosis at the permissive temperature in the presence of the translational inhibitor cycloheximide (20). p53 mutations have also been identified that uncouple transcriptional and apoptotic activities for p53 (21–24). These findings suggest the existence of a transcription-independent pathway for mediating apoptosis by p53. This apoptotic pathway has been further identified through use of an apoptosis assay in which S100 cellular extracts display a requirement for p53 protein in mediating caspase activation during in vitro incubation (25).

Apoptosis is executed by the caspase family of cysteine proteases (26–29). Caspase-9 appears to play a major role in p53-dependent apoptosis, based on loss of thymocyte radiosensitivity (30, 31) and oncogene-dependent apoptosis in embryonic fibroblasts (32) from caspase-9 null mice. Caspase-9 was also identified as an intermediate in oncogene-induced apoptosis of human fibroblasts (33). Since cytochrome c is an essential activator of caspase-9 (34), these studies suggest that apoptotic effectors of p53 may target mitochondria to induce cell death, a possibility further corroborated by the observation that p53 overexpression up-regulates Bax, which can directly mediate mitochondrial cytochrome c release (15, 35, 36). Other caspases, such as caspase-2, -7, and -8, have been found to be activated in cells undergoing p53-dependent apoptosis (37–39), but it has been unclear whether their activation is required for p53-dependent apoptosis.

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1 The abbreviations used are: MEF, mouse embryo fibroblast; FADD, Fas-associated protein with a novel death domain; DN, dominant negative; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; CMV, cytomegalovirus.
Here, using both cell-free (S100) and cell-based systems, we demonstrate that activation of caspase-8 significantly precedes caspase-3 during p53-mediated apoptosis. Caspase-8 activation is found to be essential for transcription-independent apoptosis triggered by p53 and is accompanied by formation of a newly identified 600-kDa complex containing a processed form of caspase-8 but no measurable FADD. Within cells, inactivation of FADD by a dominant negative mutant fails to interfere with the transcription-independent apoptotic process, suggesting a novel pathway for caspase-8 activation during p53-mediated apoptosis.

**EXPERIMENTAL PROCEDURES**

**Preparation of Cell-free Extracts—**p53/WT or p53/ΔN. E1A/Ras-transformed MEFs (gift of Scott Lowe, Tyler Jacks, and David Housman (5, 6)) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 10% newborn calf serum. Exponentially growing cells at ~80% confluence were either untreated or treated with 10 grays of ionizing radiation using a Gammarad 40 irradiator equipped with a 137Cs source. At various times after irradiation (1–8 h, thereby designated as 1–8-h extracts), the cells were harvested, collected by centrifugation, and washed once with ice-cold phosphate-buffered saline. To make cell-free extract from irradiated, transformed MEFs was applied to a Superose-6 HR (10/30) column (Amersham Pharmacia Biotech). The column was eluted with buffer A plus 50 µM NaCl at 0.3 ml/min, and 0.1-ml fractions were collected. Aliquots (100 µl) of the Superose-6 column fractions were analyzed by immunoblotting with antibodies against caspase-8 and FADD (StressGen). The antibody to caspase-8 (p20, L-18) was purchased from Santa Cruz Biotechnology, a peptide encompassing the active site of caspase-8. The antibody to cytochrome c was generated by injecting rats with a total of 1.2 µg of cytochrome c DNA, 0.2 µg of pBSV-LacZ (Saos2) or pEGFP (Jurkat) plus 1 µg of effector plasmids (as listed in the Figs. 3–5), using the FuGene 6 protocol (Roche Molecular Biochemicals). All effectors were controlled using empty vector transfections. For Saos2 cells, plasmids encoding caspase-8 DN (C360S), FADD DN, CrmA, caspase-9 DN (C287A), or Bel-2 were used at a ratio of 2:1 with the wild-type p53 or the p53(Gln22/2Ser22) expression construct. 30 h after transfection, the cells were fixed and stained with X-gal. Viable blue cells were discriminated from apoptotic cells using criteria previously described for this cell system (45). Apoptotic cells are smaller and round with membrane blebbing. Cells were counted from 20 randomly selected fields (200X) (46), with numbers normalized to the vector control. For Jurkat cells, 24 h after transfection, cells were harvested and washed once with ice-cold phosphate-buffered saline. The cells were then incubated sequentially with annexin V-biotin (Trevigen) and R-phycocerythrin-streptavidin (Jackson ImmunoResearch), followed by flow cytometry analysis. The transfected cells undergoing apoptosis were detected based on both green fluorescent protein expression and annexin V staining.

**Size Exclusion Chromatography Analysis of a Caspase-8-containing Complex—**Cell extracts prepared early enough that removal of endogenous p53 protein leads to efficient activation of caspase-3 (40). pcDNA3-Mch6(C287A) encodes a dominant negative mutant of human caspase-9 in which the residue 287 is altered to alanine (41). pcDNA3-FLICE(C360S) encodes a dominant negative mutant of human FADD in which the residue 360 (41). pcDNA3-Mch6(C287A) or pcDNA3-FLICE(C360S) was transiently transfected into Saos2 cells, and analyzed by immunoblotting.

**Immunoblot Analysis and Colorimetric Assay of Caspase Activation—**For examination of in vivo caspase activation, extracts prepared at various time points postirradiation were subjected to 12.5% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was then probed sequentially with antibodies against mouse caspase-8 and -3 and visualized by ECL (Amersham Pharmacia Biotech). For examination of in vitro caspase activation, samples of 10 µl of extracts prepared at 1 h following irradiation were incubated at 32 °C for various times, followed by immunoblot analyses. The antisera against caspase-8 was generated by injecting rats with a peptide encompassing the active site of caspase-8. The antibody to caspase-3 (p20, L-18) was purchased from Santa Cruz Biotechnology, a peptide encompassing the active site of caspase-8. The antibody to cytochrome c was generated by injecting rats with a total of 1.2 µg of cytochrome c DNA, 0.2 µg of pBSV-LacZ (Saos2) or pEGFP (Jurkat) plus 1 µg of effector plasmids (as listed in the Figs. 3–5), using the FuGene 6 protocol (Roche Molecular Biochemicals). All effectors were controlled using empty vector transfections. For Saos2 cells, plasmids encoding caspase-8 DN (C360S), FADD DN, CrmA, caspase-9 DN (C287A), or Bel-2 were used at a ratio of 2:1 with the wild-type p53 or the p53(Gln22/2Ser22) expression construct. 30 h after transfection, the cells were fixed and stained with X-gal. Viable blue cells were discriminated from apoptotic cells using criteria previously described for this cell system (45). Apoptotic cells are smaller and round with membrane blebbing. Cells were counted from 20 randomly selected fields (200X) (46), with numbers normalized to the vector control. For Jurkat cells, 24 h after transfection, cells were harvested and washed once with ice-cold phosphate-buffered saline. The cells were then incubated sequentially with annexin V-biotin (Trevigen) and R-phycocerythrin-streptavidin (Jackson ImmunoResearch), followed by flow cytometry analysis. The transfected cells undergoing apoptosis were detected based on both green fluorescent protein expression and annexin V staining.

**RESULTS**

**Caspase-8 Activation in p53-mediated Apoptosis—**E1A/Ras-transformed p53/WT MEFs, but not p53/ΔN MEFs, undergo rapid and extensive apoptosis following γ-irradiation (5). To examine caspase activation during p53-dependent apoptosis, E1A/Ras-transformed p53/WT or p53/ΔN MEFs were studied at various time points following γ-irradiation. For p53/WT cells, immunoblots reveal processing of caspase-8 within 4 h (p44, Fig. 1A, lanes 1–7). Activation of caspase-3 is observed about 2 h later (Fig. 1A, lanes 1–7). No caspase activation is observed in extracts from irradiated p53/ΔN cells (Fig. 1A, lanes 8–11). The temporal pattern of caspase-8 cleavage is consistent with the possibility that it may function upstream of caspase-3 during p53-dependent apoptosis.

We next examined the kinetics of caspase-8 and -3 activation in a S100 cell-free system that recapitulates key features of p53-dependent apoptosis in the absence of transcriptional activities (25). In this system, p53-dependent apoptosis is triggered by ionizing radiation, and whole cell extracts (S100) are prepared early enough that removal of endogenous p53 protein prevents subsequent activation of caspase-3 after in vivo incubation at 32 °C. As with in vivo processing, caspase-8 cleavage precedes caspase-3 by several hours (Fig. 1B, lanes 1–12). S100 extracts from irradiated, transformed p53/WT MEFs reveal no caspase-8 or -3 activation, even after 16-hours (Fig. 1B, lanes 13–16). Lack of caspase activation in p53/ΔN extracts is not due to intrinsic resistance to activation in vitro, because the addition of cytochrome c leads to efficient activation of caspase-3 in vitro without regard to the presence or absence of p53 (Fig. 1C, lanes 1–4). The cell-free system is thus also consistent with the possibility of an upstream role for caspase-8 in transcription-independent apoptosis in cells lacking p53.

**Requirement of Caspase-8 Activity for Transcription-independent Apoptosis Triggered by p53—**To test the functional requirement of caspase-8, in vitro immunodepletion was carried out at 4 °C prior to incubation at 32 °C. Immunodepletion of caspase-8 or -9 had no significant effect on levels of caspase-3 protein (Fig. 2A). Extracts containing or lacking caspase-8 or 9...
Caspase-8 in p53-dependent Apoptosis

Caspase-8 is required for caspase-3 activation in p53-dependent cell-free extracts. A, immunodepletion of caspase-8 or -9. Extracts were prepared from transformed p53−/− MEFs at 1 h after 10-gray irradiation. Caspase-8 or -9 was depleted from the extracts with a rat antiserum against mouse caspase-8 or rabbit polyclonal IgG against caspase-9. Control immunodepletion was performed with normal rat serum or rabbit IgG. The immunoblots were sequentially probed with antibodies against caspase-8, -3, and -9. The position of rat IgG heavy chain (IgG H) is indicated. B, colorimetric assays of caspase-3 activity. Extracts containing or lacking caspase-8 or -9 were incubated at 32 °C either for 12 h in the absence of cytochrome c or for 1 h in the presence of cytochrome c (200 ng). The caspase-3 substrate DEVD-pNA was then added for 30 min at 32 °C. Caspase-3 activity was determined by measuring the cleavage of DEVD-pNA. Data are mean ± S.D. from three independent experiments. p53-dependent apoptosis (black bars) was ablated by depletion of caspase-8 but was intact following depletion of caspase-9. In contrast, cytochrome c-mediated activation was ablated by depletion of caspase-9 but was intact following depletion of caspase-8.

Caspase-8 DN (C360S) or CrmA also partially protected Saos2 cells from cell death induced by wild-type p53, as indicated by a 2-fold increase in cell survival (Fig. 3B, compare lanes 1, 3, 5, and 7). This effect was specific for caspase-8, because cytochrome c was still able to trigger caspase-3 activation. In contrast, depletion of caspase-9 did not affect p53-mediated caspase-3 activation, whereas it ablated cytochrome c responsiveness (Fig. 2B, lanes 7 and 8). In all cases, antibody control immunodepletions did not affect caspase-3 activation. These data suggest that caspase-8 is essential for the p53-dependent caspase-3 activation observed in these S100 cell-free extracts.

To study the role of caspase-8 in transcription-independent apoptosis in cells, a transactivation-deficient mutant, p53(Gln22/Ser23) (40), was employed, which triggers apoptosis in multiple cell lines, including p53-deficient Saos2 cells and HeLa cells (21, 47). We first asked whether inhibition of caspase-8 activity by expression of a caspase-8 DN mutant (C360S) (41) or CrmA, a cowpox virus serpin that is a potent inhibitor of caspase-8 (44), would protect cells from p53-mediated apoptosis. As shown in Fig. 3A, overexpression of either wild type p53 or p53(Gln22/Ser23) in Saos2 cells resulted in significant cell death 30 h after transfection together with LacZ to mark transfected cells (lanes 1, 4, and 7). Expression of either caspase-8 DN (C360S) or CrmA completely blocked cell death induced by p53(Gln22/Ser23) (Fig. 3A, lanes 7–9).
FIG. 3. Caspase-8 is required for transcription-independent apoptosis induced by p53. A, Saos2 cells were cotransfected with pR5V-LacZ and expression vectors coding for either wild-type p53, p53/Gln22/Ser23, caspase-8 (C360S), or CrmA, as indicated. pcDNA3 (vector A) and pRcCMV (vector B) were used as controls. 30 h after transfection, the cells were fixed and stained with X-gal. Viable blue cells, determined by morphology, were counted from 20 randomly selected fields (200X). Data were normalized to the vector control (defined as 100% survival) and are displayed as mean ± S.E. from representative experiments done in triplicate. B, the parental and caspase-8-deficient mutant Jurkat cells were cotransfected with pEGFP and either pCMV-p53wt or pCMV-p53/Gln22/Ser23. 24 h after transfection, apoptotic cells were quantitated by annexin V-based fluorometric assays. Data are mean ± S.D. from four experiments.

that caspase-8 is an essential component in p53-induced, transcription-independent apoptosis. Moreover, wild type p53’s intermediate effects in the setting of caspase-8 deficiency suggest the existence of a second (probably transcription-dependent), caspase-8-independent death mechanism. Consistent with these observations, non-oncogene-transformed embryonic fibroblasts from caspase-8 knockout mice retain sensitivity to UV radiation, etoposide, ceramide, staurosporine, or serum starvation (49).

Caspase-8 Activation in p53-mediated Apoptosis Is Independent of FADD—Caspase-8 is an essential initiator caspase in the Fas-induced apoptotic pathway (48–51). Activation of caspase-8 in Fas-triggered apoptosis requires FADD (52), a death domain-containing protein (53, 54). Since FADD is a candidate scaffold for caspase-8 in this pathway, and in light of several studies suggesting a link between p53 and Fas in signaling apoptosis (55–58), we examined the role of FADD in p53-triggered, transcription-independent apoptosis. Saos2 cells were transfected with p53(Gln22/Ser23) along with an expression vector coding for either FADD DN, a dominant negative inhibitor that blocks Fas-induced apoptosis (43), or CrmA. Inhibition of caspase-8 activity by expression of CrmA protected Saos2 cells from apoptosis induced by p53(Gln22/Ser23); in contrast, no protective effects were observed with expression of FADD DN (Fig. 4A, compare lane 5 with lane 6). The same FADD-DN was significantly protective against Fas-induced apoptosis in these same cells and similarly had no effect on wild type p53 (data not shown). These data are consistent with the report that E1A/Ras transformed, FADD-deficient MEFs are as sensitive as wild type transformed MEFs to the induction of apoptosis by the chemotherapeutic drug adriamycin (52), a p53-mediated response (5).

Oligomerization of caspase-8 molecules is a critical step in the process of caspase-8 activation (59, 60), which generally results from their association with a caspase-8-recruiting complex, such as the death-inducing signaling complex in Fas-dependent apoptosis (61). We therefore investigated the possibility that caspase-8 may also associate in a complex during the process of caspase-8 activation (59, 60), which generally results from their association with a caspase-8-recruiting complex, such as the death-inducing signaling complex in Fas-dependent apoptosis (61). We therefore investigated the possibility that caspase-8 may also associate in a complex during p53-dependent apoptosis. S100 extracts from E1A/Ras-transformed p53−/− or p53−/− MEFs were prepared at 1 or 4 h after irradiation (1- or 4-h extracts) and fractionated by size exclusion chromatography using a Superose-6 HR column. Aliquots (100 μl) of the indicated fractions were analyzed by immunoblotting for the presence of procaspase-8, its processed form (p44), and FADD. The elution positions of molecular weight markers are indicated. C, unfractionated p53−/− extracts (untreated or 4 h after irradiation) and fraction 13 of the p53−/− 4-h extracts (shown as U, 4, or 4/F13) were analyzed by immunoblotting with an antibody against caspase-8. Procaspase-8 and its processed p44 form are indicated and demonstrate that the caspase-8 species in fraction 13 co-migrates with p44.
caspase-8 eluted as the unprocessed monomeric form (Fig. 4B, right panels, fractions 19–23), correlating with lack of caspase activation in the absence of p53 (see Fig. 1). The same state of caspase-8 was also observed in 1-h extracts from p53+/+ cells (Fig. 4B, top left panel). However, a different elution profile was observed in 4-h extracts from p53+/+ cells. Whereas unprocessed procaspase-8 molecules still migrated as monomers (Fig. 4B, middle left panel, fractions 19–23), the 44-kDa cleaved caspase-8 intermediate eluted in a larger complex of about 600 kDa (fractions 12–14). Side by side analysis of unfraccionated p53+/+ extracts (untreated or 4 h after irradiation) together with the ~600-kDa fraction (F13, Fig. 4C) confirmed that the 44-kDa species displays the same mobility as the prominent 44-kDa processed form of caspase-8 in cells and p53-dependent extracts (Fig. 4C, see also Fig. 1, A and B). These results are reminiscent of the recent finding that p35/37 processed forms of caspase-9 are associated with Apaf-1-containing apoptosome complexes (62, 63). Since FADD is known to recruit caspase-8 to the death-inducing signaling complex in Fas-induced apoptosis, we next asked whether FADD is present in this high molecular weight complex following ionizing radiation. In contrast to caspase-8, FADD eluted exclusively in small (probably monomeric) size fractions regardless of p53 status or time after irradiation (Fig. 4B, bottom panels, fractions 20–23). Although it is possible that FADD could have been released prior to or upon association of caspase-8 with the complex and thus could not be detected by our analysis, the gel filtration results are consistent with the observations that FADD is not required for caspase-8 activation in p53-dependent apoptosis, as suggested by studies with FADD DN (above) and cells derived from FADD-null mice (52).

**Requirement for Caspase-9 in p53-induced Transcription-independent Apoptosis**—Studies with caspase-9-null cells show that caspase-9 is essential for p53-dependent apoptosis (30–32). To address caspase-9’s functional role in the transcription-independent pathway examined here, we transfected Saos2 cells with p53(Gln22/Ser23) along with an expression vector encoding caspase-9 DN. Inhibition of caspase-9 activity by expression of a caspase-9 dominant negative mutant (C287A) (42) protected Saos2 cells from apoptosis induced by p53(Gln22/Ser23) (Fig. 5A), consistent with data from caspase-9−/− cells (30–32). Caspase-9 thus appears to be an essential component in the transcription-independent apoptotic pathway within cells. The dispensability of caspase-9 for caspase-3 activation in S100 extracts may reflect sufficient accumulation of active caspase-8 to mediate direct cleavage of caspase-3, which is well described in other cell-free caspase-8 apoptosis systems (64, 65). In cells, the caspase-8 signal may be significantly amplified through caspase-9 activation.

How might caspase-8 lead to activation of caspase-9 in cells? Caspase-8 could activate caspase-9 through either direct or indirect means. Caspase-9 is activated in transformed p53+/+ MEFs at 4 h following γ-irradiation, as indicated by the appearance of p35/37-processed forms of caspase-9 (Fig. 5B, top panel, lane 2). Consistent with recent reports (62, 63), we found that activated caspase-9 proteins p35/37 reside in a complex of ~700 kDa (data not shown). However caspase-9 was not detected in anti-caspase-8 immunoprecipitates from fractions containing the 600-kDa/caspase-8 complex (Fig. 5B, top panel, lane 3), whereas activated caspase-8 was present in the same precipitates (Fig. 5B, bottom panel, lane 3). It is possible that physical interactions between caspase-8 and caspase-9 could be weak or transient, thus escaping detection. However, these observations are also consistent with the possibility that activation of these two caspases occurs through formation of distinct apoptotic complexes.

One indirect means through which caspase-8 might regulate caspase-9 activation is through a Bcl-2-regulated pathway. Bcl-2 is thought to block mitochondrial permeability of apoptotic factors including cytochrome c (66, 67), an essential co-activator for caspase-9 activation (34). As shown in Fig. 5A, Bcl-2 protected against transcription-independent apoptosis triggered by the double mutant of p53 (Fig. 5A). Moreover,
immunoblot analyses with an antibody against the C terminus of Bid, a proapoptotic member of the Bcl-2 family, showed that Bid was cleaved at 3 h following irradiation of transformed p53/+/−MEFs, correlating closely with the appearance of activated caspase-8 (Fig. 5C). It has been shown that after cleavage by caspase-8, truncated Bid translocates from the cytosol to the mitochondrial membrane and induces release of cytochrome c that in turn binds to Apaf-1 and promotes activation of caspase-9 (68, 69). Together, these data are consistent with a potential role for mitochondria in activation of caspase-9 by caspase-8 in p53-dependent apoptosis, although other mechanisms, such as direct activation by proteolysis, could also be involved.

**DISCUSSION**

Our studies with both cell-free and cell-based systems demonstrate an essential role for caspase-8 in p53-mediated, transcription-independent apoptosis. Caspase-8 activation during p53-triggered apoptosis appears to be mediated by a FADD-independent mechanism, probably involving a 600-kDa complex associated with processing of caspase-8. These findings suggest a biochemical pathway for p53-triggered, transcription-independent apoptosis. In response to DNA damage and other forms of genotoxic stress, the p53 protein level and its biochemical activity are rapidly increased. Activated p53 protein may recruit or facilitate the assembly of a caspase-8-activating complex through protein-protein interactions. Caspase-8 may then target caspase-9 either through a mitochondrial pathway or by direct proteolysis, resulting in activation of caspase-3 and apoptosis.

Several lines of evidence suggest that the 600-kDa complex participates in caspase-8 activation during p53-dependent apoptosis. Initiator caspases, such as caspase-8 and -9, are in general activated by association within a complex (70). So far, the 600-kDa complex is the only larger form of caspase-8 we have identified in extracts from cells undergoing p53-dependent apoptosis, and it is clearly observed within membrane-free S100 lysates (unlike Fas/FADD-recruitment of caspase-8 (61)). The identification of partially processed caspase-8 (p44) within a complex is analogous to the recent finding that p35/p37 processing intermediates of caspase-9 are associated with Apaf-1-containing apoptosome complexes (62, 63). The 600-kDa complex may function in a similar manner for caspase-8 activation. Finally, the timing of complex formation correlates with the start of caspase-8 processing during p53-dependent apoptosis (Fig. 1A), suggesting its role in the initiation of caspase-8 activation. Further identification of components within this complex as well as the pathway leading to its formation should shed light on this alternate path to caspase-8 activation.

Caspase-8 requires caspase-9 to mediate transcription-independent apoptosis triggered by p53. This observation is consistent with a number of recent studies showing a role for caspase-9 in caspase-8-induced apoptosis of certain types of cells. Notably, Fas-induced apoptosis is significantly inhibited in Apaf-1−/−MEFs (71). In addition, the dominant negative caspase-9 mutant (C287A) has been found to block apoptosis of MCF-7 cells induced by Fas agonist antibody, TRAIL, and overexpression of TRAIL receptors DR4 and DR5 (42). Finally, hepatocytes deficient in Bid, which mediates cytochrome c release from mitochondria in response to caspase-8 cleavage, are resistant to apoptosis triggered by Fas, although caspase-8 has been activated (72). In our study, the cleavage of Bid correlates closely with the appearance of activated caspase-8, suggesting a molecular mechanism by which caspase-8 may activate the mitochondria-caspase-9-death pathway during p53-dependent apoptosis. It is currently unclear whether other intermediates, such as Smac/DIABLO (73–75) and direct proteolysis, are involved in caspase-9 activation by caspase-8 in our systems.

Immunodepletion of caspase-9 from the S100 cell-free extracts has no effect on activation of caspase-3 (Fig. 2A), demonstrating that caspase-9 is dispensable in the extracts for caspase-8-mediated activation of caspase-3. This behavior probably reflects sufficient accumulation of active caspase-8, which has been shown to be directly active caspase-3 (64, 65, 76). In vivo, the caspase-8 signal may be significantly amplified through caspase-9 activation, a step that appears to be of particular importance for p53-mediated apoptosis. It will be interesting to determine which cell types and death triggers employ such amplification via caspase-8 and to understand the biochemical basis for that need.

It may appear redundant that p53 retains both transcription-dependent and -independent pathways leading to stress-induced cell death. Yet such stress-induced apoptosis may explicitly fail if the stress were severe enough to affect transcription/translation. In such settings, a cascade involving protein-protein interactions is reminiscent of posttranslational stress response mechanisms and may be required to produce apoptotic death. It is unclear to what degree the two pathways (transcription-dependent versus -independent) are employed in different physiologic contexts, but the inhibition of caspase-8, as employed here, appears to uncouple the two and may aid in the examination of one pathway in the absence of the other.

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**REFERENCES**

1. Thompson, C. B. (1995) Science 267, 1456–1462
2. Ko, L. J., and Prives, C. (1996) Genes Dev. 10, 1054–1072
3. Levine, A. J. (1997) Cell 88, 323–331
4. Gottlieb, T. M., and Oren, M. (1998) Semin. Cancer Biol. 8, 359–368
5. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. (1993) Cell 74, 957–967
6. Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. (1994) Science 266, 807–810
7. Kuerbitz, S., Plunkett, B., Walsh, E., and Kastan, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7491–7495
8. Youle, R. J., and Rabinowitz, M. (1991) Nature 345, 345–347
9. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. (1995) Nature 362, 847–849
10. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. (1993) Nature 362, 849–852
11. Fisher, D. E. (1994) Cell 78, 539–542
12. Kastan, M. B., Fornace, A. J., and Leonard, C. J. (1995) Cancer Metastasis Rev. 14, 3–15
13. Sambati, P., Lin, J., Levine, A. J., and White, E. (1995) Genes Dev. 9, 2184–2192
14. Attardi, L. D., Lowe, S. W., Brugarolas, J., and Jacks, T. (1996) EMBO J. 15, 3693–3701
15. Miyashita, T., and Reed, J. C. (1995) Cell 80, 293–299
16. Wu, G., Burns, T., McDonald, E., Ill, J., Wang, M., Meng, R., Krantz, I., Kao, G., Gan, D.-D., Zhou, J.-Y., Muschel, R., Hamilton, S., Spinner, N., Markowitz, S. W., and El-Deiry, W. (1997) Nat. Genet. 17, 141–143
17. Polak, Y., Xia, Y., Zeswer, J. L., Kizil, K. R., and Vegelis, B. (1997) Nature 389, 300–305
18. Oda, E., Okhi, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Takeda, T., Taniguchi, T., and Tanaka, N. (2000) Science 288, 1055–1058
19. Caelles, C., Helmingberg, A., and Karin, M. (1994) Nature 370, 220–223
20. Wagner, A. J., Kokontis, J. M., and Hay, N. (1994) Genes Dev. 8, 2817–2830
21. Haupt, Y., Rowan, S., Shaulian, E., Voussden, K. H., and Oren, M. (1995) Genes Dev. 9, 2170–2183
22. Rowan, S., Ludwig, R. L., Haupt, Y., Bates, S., Lu, X., Oren, M., and Voussden, K. H. (1996) EMBO J. 15, 827–838
23. Sakamuro, D., Sabbatini, P., White, E., and Prendergast, G. C. (1997) Oncogene 15, 885–898
24. Bissonnette, N., Wasylyk, B., and Hunting, D. (1997) Biochem. Cell Biol. 75, 351–358
25. Ding, H.-F., McGill, G., Rowan, S., Schmaltz, C., Shimamura, A., and Fisher, D. E. (1998) J. Biol. Chem. 273, 28878–28883
26. Salvesen, G. S., and Dixit, V. M. (1997) Cell 91, 443–446
27. Cyns, V., and Yuan, J. (1998) Genes Dev. 12, 1551–1570
28. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281, 1312–1316
29. Wolf, B. F., and Green, D. R. (1999) J. Biol. Chem. 274, 20049–20052
30. Kuida, K., Haydar, T. P., Kano, C.-Y., Gu, Y., Taya, C., Karasuyama, H., Su,
M. S.-S., Rakic, P., and Flavell, R. A. (1998) Cell 94, 325–337
31. Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A. de la Pompa, J. L., Kagi, D., Khoo, W., Potter, J., Yoshida, R., Kaufman, S. A., Lowe, S. W., Penninger, J. M., and Mak, T. W. (1998) Cell 94, 339–352
32. Soengas, M. S., Alarcon, R. M., Yoshida, H., Giaccia, A. J., Hakem, R., Mak, T. W., and Lowe, S. W. (1999) Science 284, 156–159
33. Fearnhead, H. O., Rodriguez, J., Govek, E., Guo, W., Kobayashi, R., Hannon, G., and Lazebnik, Y. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13664–13669
34. Li, P., Nijhawan, D., Budihardjo, I., Srivinvasa, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cell 91, 479–489
35. Rosse, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B., Vincenz, C., and Dixit, V. M. (1997)
36. Chandler, J. M., Alnemri, E. S., Cohen, G. M., and MacFarlane, M. (1997) J. Biol. Chem. 272, 7357–7342
37. Jain, K., Bratton, S. B., Langlais, C., Walker, G., Brown, D. G., Sun, X.-M., and Fine, R. L. (1999) J. Biol. Chem. 274, 34924–34931
38. Yu, K., Kuo, C. J., Yuan, J., and Blenis, J. (1995) Genes Devel. 9, 1235–1246
39. Vincenz, C., and Dixit, V. M. (1997) J. Biol. Chem. 272, 6578–6583
40. Srivinvasa, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998) Mol. Cell 1, 949–957
41. Chinnaiyan, A. M., Tepper, C. G., Seldin, S., Orourke, K., Shevchenko, A., Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996)
42. Chen, X., Ko, L., Jayaraman, L., and Prives, C. (1996)
43. Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Juo, P., Kuo, C. J., Yuan, J., and Blenis, J. (1998) Curr. Biol. 8, 695–700
44. Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Bredesen, D., Green, D. R., Reed, J. C., Froelich, C. J., and Salvesen, G. S. (1998) J. Biol. Chem. 273, 1298–1299
45. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) Science 275, 1132–1136
46. Yang, J., Liu, X., Bhalia, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-L., Jones, D. P., and Wang, X. (1998) Science 275, 1129–1132
47. Li, H., Zhu, H., Xu, C.-J., and Yuan, J. (1998) Cell 94, 491–501
48. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) Cell 94, 481–490
49. Hengartner, M. (1998) Science 281, 1298–1299
50. Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A., and Gruss, P. (1998)
51. Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A., and Gruss, P. (1998) Cell 94, 727–737
52. Yin, X., Wang, K., Gross, A., Zhao, Y., Zinksi, K., Klocke, B., Roth, K. A., and Korsmeyer, S. J. (1999) Nature 400, 866–891
53. Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A., and Gruss, P. (1998) Cell 94, 727–737
54. Yin, X., Wang, K., Gross, A., Zhao, Y., Zinksi, K., Klocke, B., Roth, K. A., and Korsmeyer, S. J. (1999) Nature 400, 866–891
55. Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A., and Gruss, P. (1998) Cell 94, 727–737
56. Yin, X., Wang, K., Gross, A., Zhao, Y., Zinksi, K., Klocke, B., Roth, K. A., and Korsmeyer, S. J. (1999) Nature 400, 866–891
