p53- and Drug-Induced Apoptotic Responses Mediated by BH3-Only Proteins Puma and Noxa

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Apoptosis provoked by DNA damage requires the p53 tumor suppressor, but which of the many p53-regulated genes are required has remained unknown. Two genes induced by this transcription factor, noxa and puma (bbc3), stand out, because they encode BH3-only proteins, proapoptotic members of the Bcl-2 family required to initiate apoptosis. In mice with either noxa or puma disrupted, we observed decreased DNA damage–induced apoptosis in fibroblasts, although only loss of Puma protected lymphocytes from cell death. Puma deficiency also protected cells against diverse p53-independent cytotoxic insults, including cytokine deprivation and exposure to glucocorticoids, the kinase inhibitor staurosporine, or phorbol ester. Hence, Puma and Noxa are critical mediators of the apoptotic responses induced by p53 and other agents.

DNA damage can cause cell cycle arrest or apoptosis, and responses contribute to tumor suppression by p53 (1,2). After DNA damage, p53 imposes arrest in the G1 phase of the cell cycle by inducing expression of the cyclin-dependent kinase inhibitor p21Cip1, but the cell cycle by inducing expression of the cyclin-dependent kinase inhibitor p21Cip1, but how p53 triggers apoptosis is unresolved (1,2). Because more than 16 p53 target genes have been proposed to mediate apoptosis, it has been unclear whether any single target is critical (1,2). Noxa (3) and Puma (Bbc3) (4–6), however, merit particular attention, because BH3-only proteins [the proteins related to the Bcl-2 family only by the BH3 (Bcl-2 homology region 3) interaction domain] are essential triggers for the evolutionarily conserved path to apoptosis. Their binding to the antiapoptotic protein Bcl-2 or its close relatives launches the program, which proceeds through Bax-like family members to the proteases (caspases) that dismantle the cell (7–9). How cytotoxic drugs that kill in a p53-independent manner (for example, glucocorticoids) initiate apoptosis is also unclear, but because Bcl-2 overexpression inhibits this response (10,11), BH3-only proteins are probably critical.

Noxa-deficient mice were generated from embryonic stem (ES) cells from which noxa exons 2 and 3, which encode its two BH3 regions, had been removed (fig. S1). Correct targeting of the gene was verified (Fig. 1A), and the absence of noxa mRNA was confirmed both by reverse transcription–polymerase chain reaction (RT-PCR) analysis on irradiated thymocytes from noxa−/− mice (Fig. 1B) and by Northern blots on several tissues (Fig. 1C). Noxa does not seem to be required for normal development or physiology, because the nullizygous mice were born at the expected Mendelian frequency from noxa−/+ matings and became healthy adults. Their appearance, body weight, and organ weights were normal, as were their cellularity and the composition of hematopoietic organs (12).

The role of Noxa in stress-induced apoptosis was investigated in thymocytes (fig. S2), pre-B cells, and mature B and T cells (12), as well as in primary mouse embryonic fibroblasts (MEFs) (Fig. 1D). We tested both p53-dependent stimuli, such as the topoisomerase inhibitor etoposide and γ radiation (13,14), and p53-independent ones, such as cytokine withdrawal, the glucocorticoid dexamethasone, ionomycin (which causes calcium flux), and the phorbol ester 12-myristate 13-acetate (PMA). Noxa loss did not protect against these death stimuli in thymocytes (fig. S2), nor in the other lymphocytes (12). In contrast, noxa−/− MEFs exhibited modest but significant resistance (P < 0.02, two-tailed t test) to etoposide-induced apoptosis (Fig. 1D). In MEFs rendered sensitive to p53-mediated apoptosis by the adenovirus oncoprotein E1A (15), loss of Noxa was also protective against etoposide (Fig. 1D).

Puma-deficient mice were generated similarly from ES cells that lacked puma exons 2 and 3, which encompass the start site and the BH3 region (fig. S3). Correct targeting was demonstrated (Fig. 2, A and B), and the absence of puma mRNA was verified in cells from puma−/− mice by RT-PCR (Fig. 2C) and by Northern blot analyses (Fig. 2D). Puma also seems dispensable for normal development and health, because Puma-deficient

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mice were born at the expected frequency, had a normal appearance and normal body and organ weights, and exhibited normal cellularity and composition of hemopoietic organs (12).

The role of Puma in stress-induced apoptosis was investigated in thymocytes (Fig. 3, A and B), other lymphocytes (12), and MEFs (Fig. 3C).

Fig. 2. Molecular characterization of Puma-deficient mice. (A) Southern blot analysis on genomic DNA from Puma-deficient and littermate control mice. (B) PCR analysis with exon 2-specific primers on tail DNA from Puma-deficient and littermate control mice. The identity of the PCR product (shown by ethidium bromide staining, top) was confirmed by Southern blotting with an exon 2-specific internal oligonucleotide as a probe (bottom). (C) RT-PCR analysis on cDNAs generated from total RNA of Puma-deficient and littermate control cells (top). RT-PCR with primers specific for hprt was used as a loading control (shown by ethidium bromide staining, bottom). (D) Northern blot analysis (top) of poly (A) mRNA (4 μg) from irradiated (5 Gy) or untreated puma−/− or wild type thymocytes or spleen cells from cultured for 5 hours confirms loss of puma mRNA in the mutant mice. Full-length mouse puma cDNA was used as a probe. The filter was stripped and reprobed with a glyceraldehyde phosphate dehydrogenase (GAPDH) probe (bottom) to demonstrate the presence of RNA in all lanes.

Fig. 3. Compromised p53-dependent and p53-independent cell death responses in cells from mice that lack Puma. (A) Thymocytes from Puma-deficient mice, littermate control mouse, a p53−/− mouse, or mice that express a bcl-2 transgene controlled by the panhemopoietic vav promoter (24) were cultured for the indicated times after exposure to γ radiation. Cell viability was analyzed as in Fig. 1. Data points represent means ± SE of three independent experiments each to three animals per genotype, except for the p53−/− control (n = 1). (B) Immature (CD4−8−) thymocytes from 6- to 12-week-old mice were sorted and cultured in normal medium in the absence or presence of the indicated cell death stimuli. Viability was assessed after the indicated times by PI exclusion and flow cytometry. Data points represent means ± SE of five independent experiments and six to eight animals per genotype. (C) MEFs retrovirally transduced with the E1A oncogene and selected in puromycin were cultured in simple medium with or without serum or were exposed to etoposide. Cell viability was analyzed as in Fig. 1. Data points represent means ± SE of three independent experiments.
remains to be determined. In lymphocytes, Puma, Bim (7, 23), or both appear to have a critical role in the majority of cytotoxic responses (Fig. 4). Thus, BH3-only proteins have both overlapping and specialized roles as death initiators.

References and Notes

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Suspended Animation in C. elegans Requires the Spindle Checkpoint

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In response to environmental signals such as anoxia, many organisms enter a state of suspended animation, an extreme form of quiescence in which microscopically visible movement ceases. We have identified a gene, san-1, that is required for suspended animation in Caenorhabditis elegans embryos. We show that san-1 functions as a spindle checkpoint component in C. elegans. During anoxia-induced suspended animation, embryos lacking functional SAN-1 or a second spindle checkpoint component, MDF-2, failed to arrest the cell cycle, exhibited chromosome missegregation, and showed reduced viability. These data provide a model for how a dynamic biological process is arrested in suspended animation.

Adverse environmental conditions such as extreme temperature, decreased nutrient availability, or anoxia cause some organisms, including mammals (1), fish (2, 3), and invertebrates (4, 5), to enter a reversible state of suspended animation. For instance, nearly 100 different mammals enter into diapause, in which maternal cues induce an arrest of embryogenesis that can last for several months (7). Other examples of quiescence include hibernation (6) and estivation (7), which are programs that allow organisms to survive harsh seasonal conditions. In the zebrafish (Danio rerio), exposure to anoxia (operationally defined as <0.001 kPa O2) rapidly leads to a complete arrest of cell division, developmental progression, movement, and heartbeat. Upon reoxygenation, these biological functions are restored. Adult mammals can enter into suspended animation under conditions in which exsanguination restricts oxygen availability in the tissues (8, 9). The nematode C. elegans can enter into anoxia-induced suspended animation from any stage in the life cycle and remain suspended for several days with high viability (10).

Severe oxygen deprivation is likely to have broad physiological effects (11), and the mechanism of suspension probably involves a complex interaction of several different factors. One contributing factor may be the pas-
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