[Frontiers in Bioscience 7, d151-156, January 1, 2002]

BAX AND BH3-DOMAIN-ONLY PROTEINS IN P53-MEDIATED APOPTOSIS

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1. ABSTRACT

Activation of mitochondria-mediated apoptosis represents a major anti-tumor response of p53. One of the mechanisms for p53 to induce mitochondria-mediated cell death events is to activate genes that are directly involved in the initiation of mitochondria-induced apoptosis. Among them are Bcl-2 family members, Noxa, PUMA, and Bax. They have been shown to be direct targets in p53-mediated apoptosis. The Bax protein belongs to the multidomain Bcl-2 family, while Noxa and PUMA are BH3-domain-only proteins. This review focuses on discussing the function of these protein in p53-mediated apoptosis and how they contribute to the decision making of p53 response: growth arrest or apoptosis.

2. INTRODUCTION

Programmed cell death (apoptosis) serves to remove excess, damaged, or infected cells during development and throughout adulthood. Reducing apoptosis is involved in cancer development, while the success of cancer therapy dependents on enhancing apoptosis of cancer cells. The signaling events to apoptosis can be divided into two distinct pathways, involving either mitochondria or death receptors (1,2). In the mitochondria pathway, death signals lead to changes in mitochondrial membrane permeability and subsequent release of pro-apoptotic factors involved in various aspects of apoptosis. The released factors include cytochrome C (cyto C) (3), apoptosis inducing factor (AIF) (4), second mitochondria-derived activator of caspase (Smac/DIABLO) (5,6), and endonuclease G (7). Cytosolic cyto C forms an essential part of apoptosis complex "apoptosome", which is composed of cyto C, Apaf-1, and pro-caspase 9. Formation of apoptosome leads to the activation of caspase 9, which then processes and activates other caspases to orchestrate the biochemical execution of cells (8).

The key regulatory proteins in the mitochondria-mediated apoptotic events, the most common cell death pathway, are the Bcl-2 family of proteins, which can either promote cell survival, such as Bcl-2 and Bcl-xl, or induce cell death, including Bax and Bak. Bcl-2 and Bcl-xl appear to directly or indirectly preserve the integrity of the outer mitochondrial membrane, thus preventing cyto C release and mitochondria-mediated cell death initiation. Whereas, the pro-apoptotic proteins Bax and Bak promote cyto C release from the mitochondria (9). Different members of the Bcl-2 family share several regions of protein homology (BH domains). Some contain several BH domains and are classified as multidomain Bcl-2 members, including Bcl-2, Bcl-xl, Bax, and Bak. Others contain only the BH3 domain, a region allows interaction between Bcl-2 family members. The BH3-domain-only Bcl-2 family members, such as Bid (10,11), Bad (12), Noxa (13), and PUMA (14,15), have been shown to function as potent activators of cell death.

The p53 tumor suppressor plays a pivotal role in preventing tumorigenesis in both human and mouse. Mutations of the p53 gene or inactivation of its activity by viral and cellular proteins are the most frequent events associated with human cancers (16-18). Although p53 has been shown to participate in diverse cellular processes, including regulation of cell cycle, cellular senescence, DNA repair, cell differentiation, and angiogenesis, the most important property of p53, however, relies on its function in apoptosis, which allows p53 to selectively destroy stressed or abnormal cells and protects the organism from cancer development. The p53 protein functions as a transcription factor to regulate a set of genes mediating p53 functions. The importance of transcriptional regulation by

p53 has been demonstrated most recently by the generation of mice and embryonic stem cells with substitution of a transcriptionally inactive mutant p53 for the wild-type protein. The replacement of p53 results in loss of p53-mediated cell cycle arrest and apoptotic function in both mice and cells (19,20). The p53-dependent apoptosis is regulated, at most part, by transcriptional activation of its target genes which directly engage both death receptor and mitochondria-mediated apoptosis (21). During the past decade or so, extensive efforts have been made to identify p53 target genes involved in various aspects of apoptosis. Among the candidates, those directly involved in the mitochondrial cell death events are particularly attractive, since most of p53-initiated apoptosis appear to proceed through mitochondria (22-24). Thus, in this review, we will focus on how p53 transduces apoptosis signals to mitochondria through Bcl-2 family member proteins, such as Noxa, PUMA and Bax.

3. NOXA

Noxa belongs to the BH3-domain-only protein family. It is identified in an mRNA differential display assay when comparing expression profiles between wild type and interferon regulatory factor-I (IRF1)/p53 double null mouse embryonic fibroblasts (MEFs) after x-ray irradiation (13). A p53-binding site located at –155 to –174 of the Noxa promoter region is identified by a luciferase reporter assay. The Noxa cDNA encodes a 103-amino acid protein with two mutually related 9-amino acid sequences characteristic to the BH3 motif of the Bcl-2 family of proteins. Noxa mRNA is constitutively expressed at low abundance in the brain, thymus, spleen, lung, kidney, and testis of adult mice. X-ray irradiation of wild type MEFs induces expression of Noxa mRNA about 5-fold, with kinetics similar to those of the p53 target gene MDM2. Expression of Noxa is totally abolished in p53-deficient MEFs but not in IRF1-deficient MEFs, suggesting that x-ray-irradiation-induced Noxa expression is dependent on p53. When ectopically expressed, Noxa undergoes BH3 motif-dependent localization to mitochondria and interacts with anti-apoptotic Bcl2 family members, such as Bcl-2, Bcl-xl, but not with pro-apoptotic proteins like Bax, resulting in cyto C release from mitochondria to cytosol and the activation of caspase-9. Noxa-induced apoptosis is suppressed by co-expression of the anti-apoptotic members of the Bcl-2 family Bcl-xl or Bcl-2. Mutating one BH3 domain in Noxa results in lower pro-apoptotic activity than the wild-type protein, while the mutant carrying mutations in both BH3 domains is totally inactive. Thus, the BH3 motifs are central to Noxa's pro-apoptotic activity.

Screening for a human homologue of Noxa leads to the finding that human Noxa is identical to the ATL-derived PMA-Responsive (APR) gene, a member of the cellular immediate-early-response genes (25). Human Noxa, or APR, encodes a 54 amino acids protein containing only one BH3 motif at amino acids 29 to 37. Like its murine counterpart, the human Noxa also induces apoptosis in various cells in a BH3 motif-dependent manner. The promoter region of the human Noxa gene contains a p53-responsive element. Inhibition of Noxa by antisense oligonucleotide leads to a partial block in p53-induced apoptosis, suggesting that Noxa is likely to function cooperate with other p53 targets for the efficient induction of apoptosis.

4. PUMA

Nakano in Dr. Vousden's laboratory identifies a p53-inducible target gene PUMA (p53 upregulated modulator of apoptosis) when comparing gene expression patterns in SAOS-2 cells upon p53 induction using microarrays. PUMA encodes a BH3-domain-only pro-apoptotic protein (14). Activation of p53 in both normal and tumor cells leads to the activation of PUMA expression. Analysis of the kinetics of induction of several p53-inducible genes has shown that PUMA, like p21 and PIG3, is rapidly induced between 3 and 6 hr following p53 induction in p53-inducible SAOS-2 cells (tetracycline inducible) by the tetracycline analog doxycycline. In contrast, activation of Bax and p53R2 expression is weaker and seen later, between 6 and 12 hr after p53 induction. Rapid induction of PUMA mRNA suggested that PUMA is a direct transcriptional target of p53. Gel shift assay reveals the presence of a p53 binding site within the first intron of PUMA. Analysis of the coding potential of the PUMA transcripts reveals that differential splicing events generated different PUMA mRNAs encoding proteins PUMAα, PUMAβ, PUMAγ and PUMAδ, respectively. PUMAα and PUMAβ are BH3-containing proteins and are localized to the mitochondria, while PUMAγ and PUMAδ proteins lack the BH3 domain. Expression of PUMAα and PUMAβ inhibits cell growth and reduces colony formation more efficiently than both wild-type p53 and Bax. Mutation of the BH3 domain in PUMAα and PUMAβ completely abolished their growth inhibitory activity. The cell growth inhibition activity of PUMA is due to its function as an inducer of apoptosis. PUMAα and PUMAβ can interact with Bcl-2, while no interaction is detected between Bcl-2 and PUMAδ lacking the BH3 domain. As predicted, mutation of the BH3 domain in PUMAα and PUMAβ abolishes their ability to interact with Bcl-2. Co-expression of Bcl-2 efficiently inhibits the growth suppression effect of PUMAβ. Induction of PUMAβ results in mitochondrial release of cyto C, activation of pro-caspase 9. These studies suggest that PUMA as a p53 target induces apoptosis through mitochondria-dependent pathway.

Using a different approach, namely Serial Analysis of Gene Expression (SAGE), Yu et al. in Dr. Vogelstein's laboratory has investigated p53 response in a p53-inducible cell line (tetracycline-inducible system) derived from human colorectal cancer DLD1 cells and also identified PUMAα as a p53-inducible gene involved in apoptosis (15). The SAGE data indicate that PUMAα is induced about 10-fold in DLD1 cells after induction of p53 for 9 hr. Further analysis showed that PUMAα was induced as early as 3 hr following doxycycline withdrawal, just as is p21. PUMAα expression reaches to maximal level at 6 hr, well before the 9 hr "commitment point" for apoptosis as determined. PUMAα is predicted to encode a 193-amino

acid protein with no significant homology to other known proteins except the BH3 domain. Radiation hybrid mapping has shown that the PUMA gene is located on chromosome 19q. Using luciferase reporter assays, the same p53 consensus binding site discovered by Dr. Vousden's group has been identified. The mouse homologue of PUMA has been identified through searches of mouse EST and genomic databases. The predicted protein for the murine PUMA is 91% identical to the human protein. The sequence of the murine gene reveals the presence of a p53 binding site at a similar position as the human PUMA gene. PUMA protein co-localizes with a mitochondrial marker. Interestingly, the BH3 domain is not required for its mitochondrial localization. The lack of dependence on BH3 for mitochondrial localization is consistent with other BH3-containing proteins, though it distinguishes PUMA from Noxa, in which the BH3 domain is required for mitochondria localization. Similar to the results shown by Dr. Vousden's group, Yu et al. also shows that PUMA interacts with Bcl-2 and Bcl-xl in a BH3 dependent manner and expression of PUMA leads to reduction in colony formation. Induction of PUMA in DLD1 cells resultes in caspase 9 activation.

Although experimental evidence suggests an important role for PUMA in p53-dependent cell death, PUMA is also found induced in cells that do not undergo apoptosis in response to p53. It is possible that these cells are protected from PUMA-induced apoptosis either by independent survival signaling or the expression of anti-apoptotic genes. Like Noxa, inhibition of PUMA by antisense oligonucleotide treatment leads to only partial inhibition of apoptosis, reflecting either an incomplete inhibition of PUMA expression in these experiments or redundant role of other p53-inducible pro-apoptotic genes. A more detailed understanding of relative contribution of PUMA to p53-mediated cell death will require deletion of this gene in either human cell lines or mice. Whether PUMA acts by inhibiting anti-apoptotic protein Bcl-2/Bcl-xl or by activating pro-apoptotic BH3 domain-containing protein Bak/Bax also remains to be determined.

5. BAX

Bax represents arguably the most important pro-apoptotic Bcl-2 family protein. It contains three BH domains (BH1 to 3), but lacks the BH4 domain, which is specific to the anti-apoptotic Bcl-2 family proteins (26). The Bax gene has been shown to be a p53 target and is up-regulated in a number of systems during p53-mediated apoptosis (27). Experiments performed in Bax-deficient mice strongly support that Bax is a primary target for p53-induced apoptosis in variety of cells (28). The role of Bax in anti-cancer drug response in human cancer cells has been demonstrated using Bax knockout human colon cancer cells generated by somatic "Knock-out" techniques. Zhang et al. from Dr. Vogelstein laboratory has shown that loss of Bax resultes in either partially or complete resistance to apoptosis induced by variety of drugs, such as chemotherapeutic agent 5-fluorouracil, chemopreventive agent sulindac, and nonsteroidal anti-inflammatory drugs (29). These results have established an unambiguous role for Bax in anti-cancer therapies.

One intriguing aspect of apoptosis regulation by the members of Bcl-2 family proteins is their subcellular localization before and after death stimulation. Certain pro- and anti-apoptotic members, such as Bcl-2, Bcl-xl, and Bak, reside predominantly on mitochondria membrane and remain there during apoptosis, whereas other members such as Bax (23), Bid (10,11), and Bad (30-32) reside in the cytosol in healthy cells and translocate to outer mitochondrial membrane at the initiation of apoptosis. Current concept on Bax believes that the critical site for the pro-apoptotic activity of Bax is at mitochondria (33). The cytoplasmic Bax responds to various apoptotic stimuli by translocating to mitochondria, where it is capable of inducing cyto C release, activating caspase 9 through Apaf-1, and initiating the apoptosis cascade (34-37).

Our previous data suggest that Bax translocation is required for p53-mediated apoptosis in fibroblasts (23). The fact that a Bax mutant defective in translocation (deletion of C-terminal 21 amino acids transmembrane domain) is completely deficient in rescuing Bax-null cells from Bax-dependent apoptosis provides direct evidence that Bax translocation is required for Bax-mediated cell killing (Deng and Wu, unpublished data). Although several factors and conditions are known to trigger Bax translocation either *in vitro* or *in vivo*, including growth factor deprivation (34,38), enforced dimerization of Bax (39), and changes in cytosol pH (38), the molecular basis for this process remains to be elucidated. It is generally believed that it involves conformational changes in Bax protein that exposes its hydrophobic C-terminus containing the mitochondria transmembrane domain thus allows mitochondrial targeting. One system to study Bax translocation is based on growth factor deprivation-induced Bax translocation from the cytoplasm to the mitochondria (38). Withdrawal of growth factor causes an increase in the intracellular pH that allows the unfolding of Bax protein and the insertion of its C-terminal hydrophobic domain into the mitochondrial membrane. High pH induces an increase in the sensitivity of Bax protein towards protease treatment, enhanced recognition of Bax protein by an antibody specific for the N-terminus, and the partitioning of the protein into detergent extraction. Taken together, these results suggest that conformational changes of Bax are induced by an increase in intracellular pH.

Analysis of Bax solution structure suggests that Bax consists of 9 alpha helical structures and the C-terminal 9th helix occupies the hydrophobic pocket proposed to mediate heterodimer formation with opposing members of the Bcl-2 family (40). A conformational change involving the C-terminal helix is prerequisite to initiate mitochondrial docking of Bax. This structural change displaces the C-terminal helix from the BH3 binding pocket, promotes dimmer formation, and allows the C-terminus to interact with the mitochondrial membrane. Therefore, it appears that the C-terminal helix of Bax provides an auto-inhibitory mechanisms to prevent

exposure of the BH3 binding pocket and inhibit mitochondrial docking prior to apoptosis.

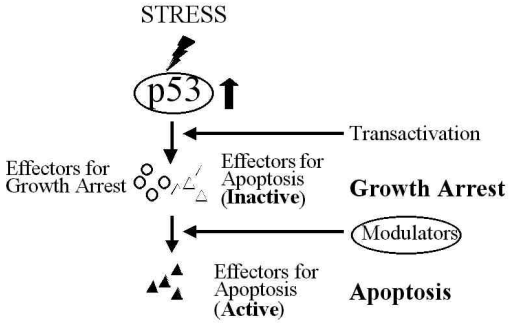

Figure 1. A sequential model for p53-mediated apoptosis vs. growth arrest. Various stress signals lead to p53 induction and transcription activation of p53 targets. As a result, effectors for both growth arrest (open circles) and apoptosis (open triangles) are induced. However, the effectors of apoptosis remain inactive and cells enter growth arrest. The presence of modulators activates pro-apoptotic factors (filled triangles) then cell death prevails.

The other critical question concerns the function of Bax in mitochondria, in particular how does Bax induces cyto C release from mitochondria? It has been proposed that Bax at mitochondria binds to the permeability transition pore complex (PTPC), a composite proteaceous channel that is involved in the regulation of mitochondrial membrane permeability (41). It has been shown that PTPC lacking Bax prepared by either immunodepletion of Bax from PTPC or purification of PTPC from Bax-deficient mice could not permeabilize membranes in response to atractyloside, a pro-apoptotic ligand of the adenine nucleotide translocator (ANT). Bax interacts directly with ANT as demonstrated by coimmunoprecipitation and in the yeast 2-hybrid system. Ectopic expression of Bax induces cell death in wild-type but not in ANT deficient yeast. Recombinant Bax and purified ANT, but neither of them alone, efficiently form atractyloside-responsive channels in artificial membranes. Therefore, it is possible that the pro-apoptotic molecule Bax and the constitutive mitochondrial protein ANT cooperate within the PTPC to increase mitochondrial membrane permeability, which triggers cyto C release. However, this may not be the only mechanism for Bax action. Most recent studies have shown that Bax resides in the mitochondria only during a brief period in the process of apoptosis. After this period, Bax leaves the mitochondria and coalesces into clusters that are adjacent to the mitochondria. In addition, the cluster formation appears to be essential for promotion of cell death by Bax (42).

6. HOW DO BAX AND BH3-DOMAIN-ONLY PROTEINS REGULATE P53-MEDIATED APOPTOTIC RESPONSE?

Although it is apparent that pro-apoptotic protein Bax and BH3-domain-only proteins Noxa and PUMA are targets of p53-mediated apoptosis, what remained unclear is whether each of these molecules contributes a part of p53-mediated response, or each of these genes responds differently to p53 activation depending on cell types and/or death signals. To understand the relationship between Bax, Noxa, and PUMA, it is worth to discuss the function of BH3-domain-only proteins in general. The BH3-domain-only proteins are best known as potent cell death inducers (43). Accumulating evidence indicates that this class of proteins functions upstream to the full multidomain Bcl-2 family members. Studies from Korsmeyer's laboratory on Bid, a BH3-domain only protein, have shown that activated Bid (tBid) triggers the homooligomerization of Bak and Bax, resulting in the release of cyto C from mitochondria. Cells lacking both Bak and Bax, but not cells lacking only one of these components, are completely resistant to tBid-induced cyto C release and apoptosis (44). Moreover, the Bax/Bak double knockout cells are resistant to various apoptotic stimuli requiring mitochondria, such as ultraviolet radiation, growth factor deprivation, endoplasmic reticulum stress, etoposide treatment, and staurosporine treatment (45). These results suggest that activation of multidomain pro-apoptotic member Bak or Bax appears to be an essential gateway to mitochondrial dysfunction required for cell death in response to diverse stimuli. This is supported by recent studies demonstrating that apoptosis induced by BH3 domain-only proteins, such as Bim, Bad, and Noxa, requires Bax or Bak (46, 47).

One of the most intriguing questions in the p53 field is how a cell makes the decision to either enter growth arrest or undergo apoptosis upon p53 induction. There are two prevailing models regarding the choice of p53 response. In the preferential gene induction model, it is proposed that p53 preferentially induces pro-apoptotic genes upon death stimulation, while the pro-apoptotic genes are not activated during p53-mediated grow arrest. In another model, it is hypothesized that p53 always induce same set of genes after activation, including genes involved in both growth arrest and apoptosis. Activation of p53-mediated apoptosis requires additional and independent signaling. Our studies on Bax translocation during p53-mediated apoptosis support the later model (23). We show that Bax is up-regulated to similar levels by p53 during either growth arrest or apoptosis. However, Bax is in cytosol during growth arrest and localizes to mitochondria during apoptosis. We further show that translocation of Bax from cytosol to mitochondria is required for p53-mediated apoptosis. We have proposed a sequential model for p53-mediated growth arrest vs. apoptosis. In this model, we propose that one of the mechanisms underlining the decision making between growth arrest and apoptosis upon p53 induction is determined by "modulator proteins". Induction of p53 results in an up-regulation of both cell growth regulating genes and pro-apoptotic factors. However, the pro-apoptotic factors, such as cytosolic Bax, remain inactive and cells enter growth arrest due to the expression of growth regulating genes such as p21 and GADD45 (Figure 1). In the presence of modulators which serve as co-activators of pro-apoptotic proteins to stimulate their apoptotic functions by inducing pro-apoptotic protein Bax translocation, cells undergo apoptosis (Fig. 1). The presence or absence of these modulators could account for the distinct responses to p53 induction in a wide variety of cells and under diverse conditions.

7. CONCLUSION

It is generally accepted that p53-mediated transcriptional activation of target genes plays a pivotal role in p53 response. The numbers of p53-responsive genes discovered increase significantly in recent years, specially aiding by the completion of human genome sequences. It also becomes increasingly clear that multiple sets of genes are involved in p53-mediated apoptosis. In addition to investigating these molecules individually, the central question for future research is how the p53 targets interplay as a network to determine the outcome of p53 response. This will require substantial effort and utilization of modern technologies, but given the importance of p53 in human cancers, we believe that this question will be answered in the near future.

8. ACKNOWLEDGEMENTS

We thank Y. Lin , N. Aithmitti, and L. Yang for discussions. This work is supported in by grants from NCI.

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Key Words: Apoptosis, p53, Bax, BH3 domain-only proteins, Review

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