BaxΔ2 Is a Novel Bax Isoform Unique to Microsatellite Unstable Tumors*

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The pro-death Bcl-2 family protein and tumor suppressor Bax is frequently mutated in tumors with microsatellite instability (MSI). The mutation often results in a “Bax negative” phenotype and generally is thought to be beneficial to the development of the tumor. Here, we report the identification of a novel Bax isoform, BaxΔ2, which is unique to microsatellite unstable tumors. BaxΔ2 is generated by a unique combination of a microsatellite deletion in Bax exon 3 and alternative splicing of Bax exon 2. Consistently, BaxΔ2 is only detected in MSI cell lines and primary tumors. BaxΔ2 is a potent cell death inducer but does not directly target mitochondria. In addition, BaxΔ2 sensitizes certain MSI tumor cells to a subset of chemotherapeutic agents, such as adriamycin. Thus, our data provide evidence that mutation and alternative splicing of tumor suppressors such as Bax are not always beneficial to tumor development but can be detrimental instead.

The instability of genomic tandemly repeated DNA sequences (microsatellites) is caused by a deficiency in the DNA mismatch repair system (1, 2). Microsatellite instability (MSI) exists in many types of cancers including colon, gastric, and endometrial cancer (3–7). MSI mutations usually result in reading frame shifts with subsequent premature termination, producing nonfunctional, truncated proteins, or nonsense-mediated decay of aberrant transcripts (8). Loss-of-function MSI mutations often occur in a number of susceptible tumor suppressors, such as Bax, and they are generally considered to be beneficial to tumorigenesis.

The pro-death Bcl-2 family protein and tumor suppressor Bax plays a critical role in tumorigenesis by regulating programmed cell death and thereby determining the chemosensitivity of certain types of tumors (9–12). The Bax subfamily includes the prototypical Baxo and several alternatively spliced isoforms such as Baxβ, ω, ψ, γ, σ, and δ (13–19). Baxo consists of six exons encoding a number of functional domains that regulate Bax subcellular localization and pro-death activity (16, 20). Baxo can form homodimers or heterodimers with Bcl-2 or other Bcl-2 family members (21, 22). Upon stimulation by death signals, Bax translocates to mitochondria, where it forms oligomers and leads to the release of cytochrome c (23–25). The known Baxα, Baxβ, and Baxψ isoforms are all capable of directly targeting mitochondria, but the underlying mechanism has yet to be elucidated (17, 26, 27).

Exon 3 of the Bax gene contains a microsatellite tract comprised of a cluster of eight guanine nucleotides (G8), which is frequently mutated in MSI tumors (7, 28–30). For instance, approximately half of MSI colon cancers and one-third of gastric and endometrial cancers contain Bax microsatellite tract mutations corresponding to a single nucleotide deletion in the Bax G8 microsatellite tract, i.e., G8 to G7 (3). This causes a reading frameshift and premature termination, usually leading to a “Bax-negative” phenotype (31–33). Although loss of Bax expression can promote tumor growth and resistance to chemotherapy (9, 10), expression levels of Bax do not always correlate with patient prognosis. Intriguingly, a better prognosis is sometimes seen in some patients with Bax-negative MSI tumors (34–36). The molecular mechanism of this apparent paradox is unknown.

Here we report the identification of BaxΔ2, a novel Bax isoform produced by the combination of a specific microsatellite mutation and an unexpected alternative splicing event. Unlike Baxα and Baxβ, BaxΔ2 does not directly target the mitochondria. As an MSI tumor-specific Bax isoform, BaxΔ2 potently induces cell death through an unconventional death pathway and sensitizes MSI tumors to a subset of chemotherapeutic agents.

EXPERIMENTAL PROCEDURES

Materials and Cell Lines—Antibodies against GFP, Bak, Bcl-2, and Bax (N20 and 6A7, both against Bax N terminus)
RESULTS

Identification of BaxΔ2 in Bax-negative MSI Cancer Cells—Expression of Bax proteins in several Bax-negative cancer cell lines including LS174T, LoVo, and DU145 (7, 38) was undetectable when analyzed by immunoblotting with commonly used anti-Bax antibodies (Fig. 1A). Consistent with the clinical observation that some patients with Bax-negative tumor had better prognosis (35, 36, 39), we noticed that some of the Bax-negative cancer cells are less aggressive than that of Bax-positive cells as analyzed in cell growth and invasion assay (Fig. 1, B–D). Interestingly, all the Bax-negative cells still expressed Bax mRNA (Fig. 1E) with various sizes of the Bax transcripts (Fig. 1F). Sequencing analyses revealed that the Bax gene isolated from these Bax-negative cells contained a single deletion in a Bax exon 3 guanine microsatellite tract, reducing G8 tracts to G7. If the G7-Bax pre-mRNA undergoes constitutive splicing, this G8 to G7 deletion should cause a reading frameshift and premature termination in the Bax transcript (Fig. 2A). However, we found that many G7-Bax transcripts actually underwent an unexpected alternative splicing that eliminated almost all of exon 2 and consequently restored the shifted reading frame at the point of the microsatellite deletion (Fig. 2A). This results in a novel Bax splicing isoform that lacks exon 2 and instead contains a frame-shifted region corresponding to a unique short peptide at the beginning of exon 3 (Fig. 2, A and B). We designated this isoform as BaxΔ2.

To determine whether the BaxΔ2 transcript can be translated as protein in cells, we generated a monoclonal antibody against the unique peptide in BaxΔ2 (Fig. 2A). We found that the BaxΔ2 antibody specifically recognized BaxΔ2 but not Baxα proteins ectopically expressed in Bax null mouse embryonic fibroblasts (MEFs) (Fig. 2, C and D). Conversely, an antibody (N20) that targets the N terminus of Baxα recognized ectopically expressed Baxα but not BaxΔ2 (Fig. 2, C and D). Thus, BaxΔ2 is a unique isoform distinct from Baxα.

BaxΔ2 Induces Cell Death without Directly Targeting Mitochondria—Bax can form homodimers or heterodimers with Bcl-2 to regulate cell death (21). To determine whether BaxΔ2 forms dimers with the Bcl-2 family proteins, we employed an in vitro binding assay, in which GST-Bcl-2 or GST-Baxα fusion proteins were used to pull down 35S-labeled BaxΔ2-GFP or Baxα-GFP in vitro. We found that both GST-Bcl-2 and GST-Baxα were able to pull down 35S-labeled BaxΔ2-GFP or Baxα-GFP in vitro (Fig. 3A). These data suggest that like Baxα, BaxΔ2 forms Bax homodimers and can also heterodimerize with Bcl-2.

Baxα is sufficient to induce cell death when it is ectopically expressed (16, 40, 41). To test whether ectopic expression of BaxΔ2 is able to induce cell death, PC3 cells were transfected with BaxΔ2-GFP, Baxα-GFP, or the control GFP. Analysis of apoptosis in GFP-positive cells revealed that the death of cells expressing BaxΔ2-GFP was significantly higher than that of cells expressing Baxα-GFP (Fig. 3C), even though the expression level of BaxΔ2 was lower than that of Baxα-GFP (Fig. 3B). However, PC3 cells have a high basal level of Baxα and perhaps other Bax isoforms. It is possible that BaxΔ2 might utilize Baxα or other Bax isoforms to induce cell death. To distinguish these
possibilities, Bax−/− MEFs were transfected with Baxα-GFP or BaxΔ2-GFP. Consistently, we found that cell death was significantly higher in Bax null MEFs expressing BaxΔ2-GFP than in cells expressing Baxα-GFP (Fig. 3, D and E). Furthermore, BaxΔ2-induced cell death is mediated by activation of caspase (Fig. 3F), and both caspase 3 and caspase 8 inhibitors, but not caspase 1 inhibitor, can effectively block BaxΔ2-induced cell death (Fig. 3G). Thus, BaxΔ2 itself is sufficient to induce cell death in a caspase-dependent manner and appears to be a more potent death inducer than Baxα.

Baxα induces cell death via directly targeting mitochondria, where it interrupts mitochondrial integrity (42). To determine the cellular localization of BaxΔ2, Bax−/− MEFs were transfected with Baxα or BaxΔ2. Cell fractionation assays revealed that Baxα predominantly distributed in the cytosolic fractions (C) (Fig. 4A), consistent with previous reports (43). By contrast, BaxΔ2 localized in the membrane fractions (M) (Fig. 4A). To determine whether BaxΔ2 targets mitochondria, we used a cell-free mitochondria targeting system. Purified tag-free Baxα or BaxΔ2 recombinant proteins were incubated with purified murine mitochondria, followed by centrifugation to separate the mitochondrial pellet from the supernatant (S). The mitochondrial pellet was further treated with alkaline to distinguish integral membrane (I) from peripheral membrane proteins (P). We found that Baxα spontaneously targeted and integrated into the mitochondria, as expected (Fig. 4B, top panel, lane 8) (23, 44). By contrast, BaxΔ2 was found almost exclusively in the supernatant (Fig. 4B, bottom panel, lane 3), indicating that BaxΔ2 did not directly target the mitochondria. The notion that BaxΔ2 and Baxα have different subcellular localizations was further supported by live imaging analysis of Bax−/− MEFs transfected with Baxα-GFP or BaxΔ2-GFP. Unlike Baxα-GFP proteins, which were evenly distributed, BaxΔ2-GFP proteins were clustered in the cytosol (Fig. 4C). More importantly, immunostaining analysis of BaxΔ2 revealed that BaxΔ2 did not co-localize with mitochondria under a resting condition (Fig. 4D, bottom panels).

To determine whether BaxΔ2 may affect mitochondrial functions, we measured the release of cytochrome c from mitochondria in Bax−/− MEFs expressing Baxα or BaxΔ2. We found that like Baxα, ectopic expression of BaxΔ2 also triggered the release of cytochrome c (Fig. 4E). Interestingly, the loss of mitochondrial membrane potential in the BaxΔ2 transfected cells...
was partially blocked by caspase 8 inhibitor but not caspase 3 inhibitor (Fig. 4F). These data suggest that BaxΔ2 affects mitochondrial functions without directly targeting mitochondria, and the underlying mechanism is partly mediated by caspase 8.

**BaxΔ2 Is an MSI Tumor-specific Bax Isoform**—The generation of BaxΔ2 needs the unique combination of a microsatellite deletion and atypical exon 2 alternative splicing. We used a panel of MSI tumor cell lines to determine the relationship between the production of BaxΔ2 and the Bax gene microsatellite status (G7, G8, or G9). RT-PCR results revealed that all BaxΔ2-positive cells had a single guanine nucleotide deletion at the exon 3 microsatellite site, i.e., a G7 status, although not all G7 MSI tumor cells had detectable BaxΔ2 transcripts (Table 1). Importantly, BaxΔ2 transcripts were also detected in some G7 MSI primary tumors (Table 1). Analysis of RNA samples isolated from human prostate cancer and colon cancer revealed that two of five colon samples and three of five prostate tumor samples contained a G7 microsatellite tract. Among these samples, one colon and one prostate tumor also had BaxΔ2 transcripts (Table 1). It is possible that in some G7 MSI tumor cells or primary tumors the copy number of BaxΔ2 transcripts is too low to be detected, or the MSI mutation is necessary but not sufficient to produce BaxΔ2. Future studies are needed to distinguish these possibilities. Taken together, these data indicate that BaxΔ2 exists in primary tumors in addition to MSI tumor cell lines.

To determine whether the alternative splicing machinery involved in generation of BaxΔ2 is MSI tumor-specific, we designed a Bax mini-gene GFP assay (Fig. 5A). The mini-gene was constructed by inserting part of the Bax genomic DNA sequence, including exons 1, 2, and 3 and the 5′ end of exon 4, into the 5′ end of a GFP reporter vector, with either a G7 or G8 microsatellite tract in exon 3 (G7 or G8 mini-gene) (Fig. 5A). The mini-genes were transfected into two prostate cancer cell lines, microsatellite G7 BaxΔ2-positive 104-R1 cells (Fig. 5B), or G8 BaxΔ2 negative PC3 cells (Fig. 5C). In the case of constitut-
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The splicing machinery involved in Bax splicing event occurred at a higher efficiency in the G7 Bax mini-gene but not in the G8 mini-gene (Fig. 5A). We found that in G7 BaxΔ2-positive 104-R1 cells, both constitutive splicing and alternative splicing occurred, but only the G7 mini-gene product was recognized by the anti-BaxΔ2 antibody (Fig. 5B). The same results were also observed in G8 BaxΔ2 negative PC3 cells (Fig. 5C).

Next we examined the propensity of cells to splice endogenous BaxΔ2 transcripts versus Baxα transcripts. Transcripts from Baxα-positive PC3 and BaxΔ2-positive 104-R1 cells were amplified by PCR using primers surrounding exon 1 and the 5’ end of exon 4 (Fig. 5D). We found that both cell lines produced endogenous transcripts in which exon 2 was spliced, although this splicing event occurred at a higher efficiency in the G7 BaxΔ2-positive 104-R1 cells (Fig. 5D). It is likely that the trans splicing machinery involved in BaxΔ2 generation exists in both Bax G7 and G8 tumor cells with various splicing efficiencies.

However, the splicing itself without a G7 microsatellite mutation is unable to produce BaxΔ2. Thus, BaxΔ2 is an MSI tumor-specific Bax isoform.

BaxΔ2 Selectively Sensitizes MSI Tumor Cells to a Subset of Chemotherapeutic Agents—To determine whether BaxΔ2-positive cells are more sensitive to cell death stimuli, G7 BaxΔ2-positive 104-R1 cells and G8 BaxΔ2-negative PC3 cells were treated with adriamycin (topoisomerase II inhibitor), bortezomib (proteasome inhibitor), and tunicamycin (endoplasmic

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**FIGURE 3. BaxΔ2 is a potent cell death inducer and retains Bax dimerization properties.** A, the interaction between purified GST-Baxα (or GST-Bcl-2) and [35S]Met-labeled Baxα-GFP (or [35S]Met-labeled BaxαΔ2-GFP) were analyzed in an in vitro binding assay. B-E, immunoblotting analysis with anti-GFP antibody (B and E) and cell death assay (C and D) of PC3 cells (B and C) or Baxα−/− MEFs (D and E) transfected with Baxα-GFP and BaxΔ2-GFP. F, caspase assay of PC3 cells transfected same as B and C, G, cell death assay of PC3 cells transfected with BaxΔ2-GFP in the presence of pan-caspase inhibitor (z-VAD), caspase 3 inhibitors (DEVD), caspase 8 inhibitor (IETD), or caspase 1 inhibitor (YVAD) 50 μM each as indicated. **, p < 0.001. inh., inhibitor.

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**FIGURE 4. BaxΔ2 is localized in membrane fraction but does not target mitochondria.** A, BaxΔ2/Mefs transfected with Baxα or BaxΔ2 for 16 h, then fractionated, and analyzed for Bax expression by immunoblotting using anti-Baxα antibody (N20) and anti-BaxΔ2 antibody (2D4). Lanes C, cytosol fraction; lanes M, membrane fraction. B, mitochondrial targeting assay (41). Purified tag-free Baxα or BaxΔ2 proteins were incubated with purified murine mitochondria, followed by alkaline stripping to distinguish integral membrane (l-Memb) from peripheral membrane (P-Memb) proteins. C, live green fluorescence imaging analysis of BaxΔ2−/− MEFs expressing GFP, Baxα-GFP, or BaxΔ2-GFP. D, BaxΔ2−/− MEFs were transfected with BaxΔ2 and then treated without (top panel) or with (bottom panel) adriamycin (4 μg/ml). The cells were stained with MitoTracker (red), fixed, and followed by immunostaining with anti-Baxα antibody (green); nuclei were stained with DAPI. E, BaxΔ2−/− MEFs were transfected with GFP, Baxα-GFP, or BaxΔ2-GFP. The cytosol fractions were collected and analyzed by immunoblotting with anti-cytochrome c antibody. F, BaxΔ2−/− MEFs transfected with GFP or BaxΔ2-GFP and then treated with or without caspase 8 inhibitor (IETD-fmk, 50 μM) or caspase 3 inhibitor (DEVD-fmk, 50 μM). The cells were stained with MitoTracker, and mitochondrial membrane potential (MMP) losses were quantified as the percentages of non-MitoTracker staining cells over total transfected green cells. **, p < 0.05. Ctrl, control; Cyto c, cytochrome c; inh., inhibitor.
The cytotoxicity assays revealed that G7 BaxΔ2-positive cells were more sensitive to adriamycin but less sensitive to bortezomib or tunicamycin (Fig. 6A), suggesting that BaxΔ2 may be selectively involved in a certain type of cell death pathway. Interestingly, BaxΔ2-positive cells (104-R1 and LS174T) were more sensitive to adriamycin than Bax-positive cells (PC3 and SW1116) (Fig. 6B) but were not sensitive to another topoisomerase II inhibitor etoposide (Fig. 6B) (45, 46). The sensitivity and selectivity to different chemotherapeutic agents were not the result of up-regulation of BaxΔ2 and Baxα or Bcl-2 protein levels (Fig. 6C). To exclude the possibility that the cell type differences may count for the different chemosensitivities, BaxΔ2-GFP-transfected PC3 cells were treated with etoposide or adriamycin. We found that BaxΔ2 selectively sensitized the cells to adriamycin- but not etoposide-induced cell death (Fig. 6D). Thus, BaxΔ2 may determine the chemoselectivity through distinct death pathways in the MSI tumor cells.

**DISCUSSION**

Baxα and other Bax isoforms induce apoptosis by targeting mitochondria (17, 27, 42). In contrast, BaxΔ2 induces apoptosis without directly targeting mitochondria (Figs. 3 and 4). Structurally, BaxΔ2 is almost identical to Baxα except for the deletion of 10 amino acids generated by the frameshift within this region (Fig. 2A). The deletion of amino acids 13–38 may be responsible for the inability of BaxΔ2 to target mitochondria, because the amino acids 16–35 are part of the first α-helix of Baxα that is required for its mitochondrial targeting (27). BaxΔ2 localizes in nonmitochondrial membrane fractions, not the cytoplasm (Fig. 4A). It is possible that the addition of the unique short peptide in BaxΔ2 (Fig. 2B) could target it to a yet to be identified membrane compartment. Interestingly, BaxΔ2-induced cell death is accompanied by the release of cytochrome c (Fig. 4E) and loss of mitochondrial membrane potential, which can be partially blocked by caspase 8 inhibitor but not caspase 3 inhibitor (Fig. 4F). Future studies are needed to determine the exact subcellular target of BaxΔ2 and the underlying mechanism by which BaxΔ2 induces cell death.
Like the prototypic Baxα, BaxΔ2 is involved in determining the sensitivity of tumor cells to chemotherapeutic agents (Fig. 6). However, BaxΔ2 sensitizes G7 MSI tumor cells to adriamycin but not etoposide, although both are topoisomerase II inhibitors (Fig. 6). It is likely that BaxΔ2 may be involved in an Adriamycin-induced nonmitochondria death pathway rather than promoting the inhibition of topoisomerase II by adriamycin. Interestingly, a side effect of adriamycin treatment is severe cardiotoxicity, which is somewhat attenuated in etoposide-presumably nonsense-mediated decay of the transcript (Fig. 2A). A frameshift will result in a premature termination codon and inhibiting the alternative splicing to delete exon 2 (Fig. 5), without Table 1). Even though G8 MSI tumor cells are capable of carrying out the alternative splicing to delete exon 2 (Fig. 5), without the guanine nucleotide deletion in the microsatellite tract, the frameshift will result in a premature termination codon and presumably nonsense-mediated decay of the transcript (Fig. 2A). Although expression levels of BaxΔ2 are quite low in MSI tumor cells (Fig. 6), because it is a potent death inducer when overexpressed and therefore is likely detrimental to the tumors, it may be possible to up-regulate BaxΔ2 expression by inducing the transition of G8 to G7 or modulating the splicing machinery to enhance exon 2 splicing, thereby triggering or sensitizing cell death in certain type of MSI tumors. Future studies are needed to explore these possibilities.

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