Experimental Cell Research 359 (2017) 342–355

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

The functional domains for BaxΔ2 aggregate-mediated caspase 8-dependent cell death

Adriana Mañas[a], Sheng Wang[b], Adam Nelson[a], Jiajun Li[a], Yu Zhao[a], Huaiyuan Zhang[a], Aislinn Davis[a], Bingqing Xie[c], Natalia Maltsev[b], Jialing Xiang[a,*]

[a] Department of Biology, Illinois Institute of Technology, Chicago, IL 60616, USA
[b] Human Genetics Department, Computation Institute, University of Chicago, Chicago, IL 60637, USA
[c] Department of Computer Science, Illinois Institute of Technology, Chicago, IL 60616, USA

ARTICLE INFO

Keywords:
Bax
BaxΔ2
Caspase 8
Apoptosis
Aggregation
Death domain

ABSTRACT

BaxΔ2 is a functional pro-apoptotic Bax isoform having alterations in its N-terminus, but sharing the rest of its sequence with Baxα. BaxΔ2 is unable to target mitochondria due to the loss of helix α1. Instead, it forms cytosolic aggregates and activates caspase 8. However, the functional domain(s) responsible for BaxΔ2 behavior have remained elusive. Here we show that disruption of helix α1 makes Baxα mimic the behavior of BaxΔ2. However, the other alterations in the BaxΔ2 N-terminus have no significant impact on aggregation or cell death. We found that the hallmark BH3 domain is necessary but not sufficient for aggregation-mediated cell death. We also noted that the core region shared by Baxα and BaxΔ2 is required for the formation of large aggregates, which is essential for BaxΔ2 cytotoxicity. However, aggregation by itself is unable to trigger cell death without the C-terminus. Interestingly, the C-terminal helical conformation, not its primary sequence, appears to be critical for caspase 8 recruitment and activation. As BaxΔ2 shares core and C-terminal sequences with most Bax isoforms, our results not only reveal a structural basis for BaxΔ2-induced cell death, but also imply an intrinsic potential for aggregate-mediated caspase 8-dependent cell death in other Bax family members.

1. Introduction

The pro-apoptotic Bcl-2 family member Bax plays a crucial role in anti-tumorigenesis [1–4]. Previous studies have shown that Bax possesses a significant *"plasticity"*, as several functional Bax isoforms have been identified [5–14]. The most prominent form of the Bax subfamily, Baxα, is ubiquitously expressed in most mammalian cells and has been well studied. Baxα has 6 exons that code for 192 amino acids. Its secondary structure is comprised of 9 alpha (α) helices with small unfolded regions in between to form a globular tertiary structure [15]. Several Baxα functional domains have been identified based on their similarities with other Bcl-2 family members. These include Bcl-2 homology domains: BH3 located in exon 3, BH1 in exon 4, and BH2 in the boundary region between exons 5 and 6 [1,3,4,16]. The BH3 domain in all pro-death members, including the Bax subfamily, plays an essential role in inducing apoptosis. Structurally, the Baxα BH3 domain is part of a hydrophobic pocket formed mainly by helices α2, α5, and α6 encoded by exons 3, 4, and 5, respectively. The pocket is covered by helix α1, which is encoded mostly by exon 2 [15,17–20].

The first 20 amino acids of the Bax N-terminal region, which do not share homology with any other member of the Bcl-2 family, conform a special domain known as Apoptosis-Regulating Targeting (ART). ART was initially believed to be a part of the mitochondrial targeting sequence, but later it has been shown to be not essential for mitochondrial targeting but critical for preventing Baxα from becoming activated in the absence of death signals [8,9,21–25]. The underlying mechanism is not fully understood, but it is believed that ART is responsible for maintaining the inactive monomeric form of Baxα proteins. Upon stimulation by cell death signals, Baxα monomers undergo conformational changes, form oligomers, and target mitochondria [26–33]. Although the exact nature of the conformational changes is controversial, it is generally accepted that the death signal-induced exposure of the BH3 domain is critical for Baxα activation. It is also known that helices α2 to α6, encoded by exons 3–5, are required for Baxα oligomerization [29,33–36], and helix α1 is essential for Baxα translocation to mitochondria [22,37–39]. Recently, spectroscopic and scattering techniques have been used to identify the structures of the active Baxα monomer, dimer, and tetramer. These findings further support the concept that the displacement of helix α1 from the BH3-domain containing hydrophobic pocket is the first step for Baxα

* Correspondence to: Department of Biology, Illinois Institute of Technology, 3101 South Dearborn Street, Chicago, IL 60616, USA.
E-mail address: xiang@iit.edu (J. Xiang).

http://dx.doi.org/10.1016/j.yexcr.2017.08.016
Received 14 June 2017; Received in revised form 7 August 2017; Accepted 8 August 2017
Available online 12 August 2017
0014-4827/

activation, leading to oligomerization and mitochondrial translocation [34].

The exact function of the Bax C-terminal region remains controversial. The Baxα C-terminal region has a transmembrane domain (TM) contained in helix α9 [15,34,40,41], which has been reported to play a critical role in targeting [2,31,42,43], anchoring, and penetrating the mitochondrial membrane [40,44–46]. However, it has also been shown that alteration or deletion of the C-terminal region has little influence on the ability of Baxα to target mitochondria [22,25,47–50] or its pro-apoptotic activity [25,47,48,50]. Furthermore, although Baxβ, Baxσ, Baxε and Baxω isoforms have different C-termini, they all can release cytochrome C and trigger mitochondrial cell death in the same way as Baxα [5,6,12,14].

BaxΔ2 and its subfamily members are a group of unique functional Bax isoforms initially identified in patients with a family history of mismatch repair deficiency [13,51–53]. BaxΔ2 has a guanine deletion in exon 3 (G8 → G7), which causes a frameshift and pre-mature termination of translation. However, an alternative splicing event removes most of exon 2 and restores the reading frame, resulting in a full-length functional isoform (BaxΔ2) with 10 new amino acids between the alternative splicing site and the point of the deletion. The rest of the BaxΔ2 sequence is the same as Baxα [13,51,53].

BaxΔ2 is pro-apoptotic and still able to form dimers with Baxα and Bcl-2 [13]. However, BaxΔ2 is unable to target mitochondria due to the lack of helix α1 [13,22,37–39]. Instead, BaxΔ2 forms cytosolic aggregates, promoting apoptosis by activating caspase 8 [13,51,53]. However, the functional domain(s) for the aggregation and activation of caspase 8 were unknown. To understand the underlying molecular mechanism of BaxΔ2-induced apoptosis, we used computational structural modeling and cell-based approaches to analyze the functional domains that are responsible for its aggregation and cytotoxicity. Our results reveal that, apart from the impairment of mitochondrial translocation, the changes in the N-terminal of BaxΔ2, when compared to Baxα, seem to have no significant effect on its aggregation and cytotoxicity. The lack of helix α1 exposes the core of the protein and promotes BaxΔ2 to form cytosolic aggregates, which activate caspase 8 via the C-terminus in a helical structure-dependent manner to trigger cell death.

2. Materials and methods

2.1. Cloning

All BaxΔ2 constructs indicated in the text were generated using either Baxα or BaxΔ2 as a template for PCR-based cloning. The constructs were cloned by insertion of the corresponding PCR product in a pcDNA3.1 vector between *EcoRI* and *KpnI* sites. Each construct was in-frame tagged with a green fluorescence protein (GFP) at its C-terminus in the *HindIII* site. There is a 15-base pair linker between the construct and GFP gene. Deletions (Δ) were made and numbered according to the BaxΔ2 amino acid sequence. BaxΔ2[Δ13–21/G7] is a BaxΔ2 without the 10 new amino acids, while BaxΔ2[R13-21/G8] has the same 10 amino acids as Baxα (R representing replacement of the amino acids). An extra base pair was added to the latter to avoid a frameshift, which makes this construct equal to Baxα without exon 2. BaxΔ2[ARTΔ13–21/G7] is BaxΔ2 with a full-length ART sequence, but deletion of the 10 amino acids. BaxΔ2[Δ1–21] is BaxΔ2 with deletion of exon 1 and the 10 amino acids. This construct is basically Baxα without the first 2 exons. BaxΔ2[Δ13–61] is BaxΔ2 with complete deletion of exon 3, which could also be interpreted as Baxα without exons 2 and 3. An extra base pair was also added to this construct to avoid a frameshift. BaxΔ2[ΔBH3] is BaxΔ2 without the BH3 domain. BaxΔ2[Δ62–175] is BaxΔ2 without exons 4, 5, and 6. BaxΔ2[Δ110–175] is BaxΔ2 without exons 5 and 6. The beginning of the exon 5 sequence was retained to maintain the integrity of helix α5. BaxΔ2[Δ141–175] is BaxΔ2 without exon 6. C-terminal point-mutants were generated using the Transformer™ Site Directed Mutagenesis Kit (Clontech, Takara). All constructs were validated by DNA sequencing.

2.2. 3D structure modeling and computational analyses

Structural models for BaxΔ2 and its mutant constructs were created using RaptorX [54–56] and I-TASSER [57–59] protein tertiary structure prediction server. Specifically, RaptorX first predicts the secondary structures, solvent accessibility, and disordered regions for the query sequence and then utilizes this information to search for templates through which to construct the 3D model of the query by Modeler [60] or Rosetta [61]. I-TASSER predicts the 3D model of the query sequence through four general steps: threading template identification, iterative structure assembly simulation, model selection, and refinement. Models obtained by different methods were compared and the structural similarity was determined as TM score (range between 0 and 1). Models of Baxα were also created and compared with the known structure as control. The disorder probability of the models was determined using PrDOS [62]. Analysis of conservative motifs between the C-terminal regions of BaxΔ2 and BaxΔ2ω was done with the assistance of the ClustalW server [63]. Helicity probability analysis was performed using NetSurfP 1.1 [64] and GOR4 [65]; helicity probability values from both software programs were averaged, then values of BaxΔ2 were subtracted from each mutant construct's values before plotted.

2.3. Cell culture and transfection

The Bax negative colon cancer HCT116 subline cell line was generated as described previously [53]. All cells were cultured in Dulbecco's Modification of Eagle's Medium supplemented with 10% FBS. For transfection, cells were split in a 6-well plate, allowed to grow until 60% confluence, then transfected with the appropriate constructs, as indicated in the text, using Lipofectamine® 3000 Reagent (Invitrogen). Cells were then incubated for the time corresponding to each experiment. When required, cells were treated with 40 μM of Etoposide for 4 h.

2.4. Immunostaining and imaging

Bax-deficient MEFs were transfected on glass cover slides in a 6-well plate, incubated at 37 °C for 16 h, then fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X100 in Phosphate Buffered Saline (PBS). When required, cells were incubated overnight at 4 °C with a primary antibody against Tom20 (Santa Cruz Biotechnologies, 1:100 dilution) or against caspase 8 (Cell Signaling, 1:100 dilution), followed by secondary antibody Alexa Fluor® 594 (Life Technologies, 1:200 dilution) for 1 h at room temperature. Nuclei were stained with DAPI during the mounting process with ProLong® Gold antifade reagent (Invitrogen). Fluorescent imaging was performed using a Keyence BZ-X710 All-in-One Fluorescence Microscope equipped with BZ-X Viewer software. Images were analyzed using BZ-X Analyzer software and ImageJ 1.48. All cells were imaged using Z-stack, in which at least 30 sections separated by 0.2–0.3 μm were taken and composed by full focus. Of note, the images for mitochondrial co-localization are from individual sections, not composed. For the co-localization analysis of Caspase 8, main images correspond to individual sections and the 3D analysis was performed using the XYZ Slicing tool. Approximated size of aggregates was estimated by measuring a total of 30 random clearly individual aggregates from 3 representative cells for each construct.

2.5. Cell death and caspase assays

Bax negative HCT116 subline cells were transfected with GFP-tagged BaxΔ2 derived constructs and incubated for 24 h in the absence