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The alpha-5 helix of Bax is sensitive to ubiquitin-dependent degradation

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ABSTRACT

The pro-apoptotic protein Bax is instable in many cancer cells but the mechanism of Bax degradation remains unclear. Four different lengths of deductive Bax degradation sensitive (BDS) sequences within BH3–BH1 region, BDS-1 (Bax 67–124), BDS-3 (Bax 74–107), BDS-5 (Bax 67–107), and BDS-7 (Bax 74–124), were tested for the susceptibility to ubiquitin-dependent degradation. Both BDS-1 and BDS-7 which contain the α5 helix, a putative pore-forming domain of Bax, are sensitive to proteasome-dependent degradation and ubiquitin-conjugation. The Bax α5-deletion mutant (Bax-Δα5) was stable and also maintained its apoptosis-inducing ability. Deletion of helices α1 and part of α2 (Bax-Δ1–66) or helices α3 and α4 (Bax-Δα3,4) did not affect the sensitivity to degradation. However, Bax-Δ1–66 mutant was not able to induce apoptosis. Thus, we propose that the α5 helix of Bax is sensitive to ubiquitin-dependent degradation. Moreover, Bax mutant retains its pro-apoptosis ability when the α5 helix is deleted.

Proteins of the Bcl-2 family play important roles in the cell fate decision. The balance between the anti-apoptotic proteins such as Bcl-2 and the pro-apoptotic proteins such as Bax is critical for cells to live or to die. Mechanisms that increase levels of the anti-apoptotic Bcl-2-like proteins or decrease the amount of the pro-apoptotic Bax-like proteins tip the balance in favor of survival. Low levels of Bax or the higher ratios of Bcl-2/Bax have been used either as predictors of clinical outcome for the treatment or as poor prognostic markers for cancer patients [1–5]. Bax is an instable protein and its degradation activity increases in many cancer cells which is associated with a worse clinical prognosis [6–9]. However, the mechanism by which Bax is sensitive to degradation is poorly understood.

The structure of Bax consists of nine α helices, with eight of these, being amphipathic, clustered around one central hydrophobic α helix (α5). The functional domains of Bax, BH1 (α4 and α5 helices), BH2 (α7 and α8 helices), and BH3 (α2 helix) domains, form a hydrophobic groove that is occupied by its own C-terminal transmembrane domain (TMD) [10]. It has been reported that the α1 helix is essential for Bax to interact with the BH3-only proteins, such as Bid and PUMA [11], or with the anti-apoptotic protein Bcl-xL [12], playing an essential role in activating Bax. The helix α2 encompasses the BH3 domain of Bax and regulates interactions within the family [13]. The central α5 and α6 helices and regions of the amino-terminus form a hairpin structure, which has been termed as the putative pore-forming domain of Bax and contributes to the regulation of Bax mitochondrial translocation, membrane binding, and interaction with Bcl-xL and Bax itself [12–14]. The C-terminal TMD (α9 helix) of Bax was originally identified as a transmembrane domain which is responsible for Bax translocation to the mitochondrion [10,15,16]. A recent study proposed that the Bax C-terminal deletion mutant could induce apoptosis but appeared as a monomer, demonstrating that Bax C-terminus is involved in the stabilization of Bax oligomers [17].

Previously, we have observed that Bax degradation in the malignant B-cells during the treatment with TRAIL. Bax degradation can be blocked by a proteasome inhibitor Bortezomib. In a cell-free system, Bax degradation can be prevented by a truncated form of BH3-only protein Bid, tBid [8]. Other study found that tBid, but not the full-length Bid, is also a short-lived protein which degrades rapidly during treatment with TNF and cycloheximide [18].

It is of note that the degradation of pro-apoptotic proteins, such as Bax and tBid, happens after the cell received the apoptotic signal and could diminish the apoptotic process within the cell. The aim of this study is to identify whether the Bax degradation sensitive domain is localized within a region which is exposed during the apoptotic process.

Materials and methods

Cell lines, cell culture, and gene transfection. The human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2. The Fugene-6 transfection reagent (Roche Applied Science) was used for the transient transfection.

Plasmid constructs. All Bax mutant constructs were established by PCR using the pEGFP/C3-Bax as a template. The primers which were used to amplify Bax and its mutants cDNA, and subclone them into the pCMV-Tag-2B vector are: gagctc gccacc atg gaattc atg gac ggg tcc ggg gag c (Bax-Tag-S) and attatcaa ctcgag tca gcc cat ctt ctt cca g (BaxTM-Tag-As) for Bax; gagctc gccacc atg ggatcc gaattc ggg gac gaa ctg gac agt aac (T7-BDA1) and BaxTM-Tag-As for Bax-Δ1–66; Bax-Tag-S and Bax-ΔTMD-AS attatcaa ctcgag ggatcc tca ctg cca cgt ggg cgt ccc for Bax-ΔTMD; Bax-Δ1–33-S

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gagctc gccacc atg gaattc cga gca ggg cga atg ggg and BaxTM-Tag-As for Bax-Δ1–33. All PCR products of Bax were cloned into the pGEM-T-easy vector (Promega), and the sequences were confirmed. Bax-Δα3–4 deletion mutant was constructed with primers ggc ggc ggc gac atg ttt tct gac ggc (Bax-Δα3,4-S) and gtt act gtc cag ttc gtc c (Bax-Δα3,4-AS). Bax-Δα5 deletion mutant was constructed with primers Bax-Δα5-S ggc ggc ggc ccg gaa ctg atc aga acc and Bax Δα5-AS cca gtt gaa gtt gcc gtc. The pGEM-T-easy-Bax construct was a template for amplifying both of Bax-Δα3–4 and Bax-Δα5 using a modified Exsite PCR-based site-directed Stratagene Mutagenesis Kit. Bax and its mutant constructs in the pGEM-T-easy vector were digested with EcoRI and XhoI, and ligated into the sties between EcoRI and XhoI of pCMV-Tag-2B (Stratagene) which has an N-terminal flag-tag fused with expressing protein.

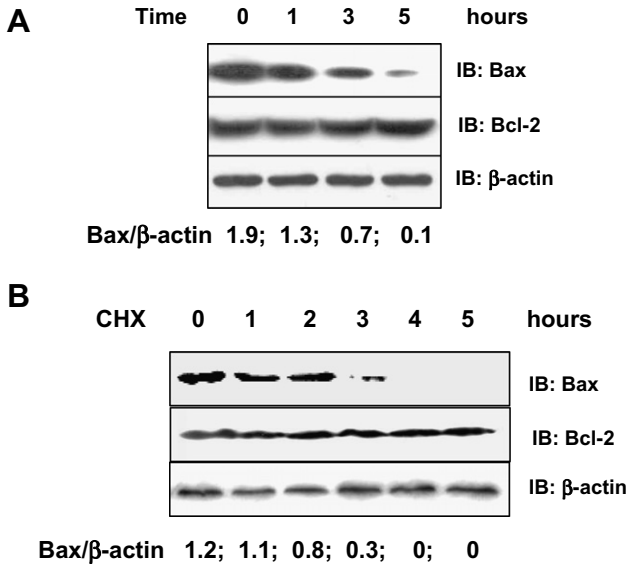

Fig. 1. Bax degradation activity in 293T cells. (A) Bax degradation in a cell-free system. The post-nuclear supernatant from 293T cells was incubated in the presence of ubiquitin and ATP regeneration system. Proteins were collected hourly for Western blotting. (B) 293T Cells were treated with 100 μg/ml CHX for up to 5 h. Bax and Bcl-2 protein levels were determined by Western blotting. The anti-Bax antibody (clone 2D2, R&D Systems) was used at 1:1000 dilution, the monoclonal anti-Bcl-2 antibody (clone 100, Santa Cruz) was used at 1:200 dilution, and the anti-β-actin antibody was used at 1:10,000 dilution. IB, immunoblotting. The ratio of Bax/β-actin was measured by densitometry.

The primers which were used to amplify Bax mutants cDNA and subclone them into the pEGFP-C1 vector are: BDA1 (attatcaa ctcgag ct ggg gac gaa ctg gac agt aac) and BDB1 (attatcaa ggatcc tca ggc ctt gag cac cag ttt gc) for EGFP-BDS-1; BDA2 (atta tcaa ctcgag ct atg gag ctg cag agg atg) and BDB2 (attatcaa ggatcc tca cca gtt gaa gtt gcc gtc) for EGFP-BDS-3; BDA1 and BDB2 for EGFP-BDS-5; BDA2 and BDB1 for EGFP-BDS-7; and BDTM (attatcaa ctcgag ct tgg cag acc gtg acc atc) and BDTMB (attatcaa ggatcc tca gcc cat ctt ctt cca g) for EGFP-TMD.

PCR products of EGFP-BDS-1, EGFP-BDS-3, EGFP-BDS-5, EGFP-BDS-7, and EGFP-TMD (EGFP-BDS-9) were digested with XhoI and BamHI, and cloned into the site between XhoI and BamHI of the pEGFP-C1 vector. The sequences of the constructs in pEGFP-C1 vector were confirmed.

Determination of Bax degradation and the lifespan of Bax protein. For detecting Bax degradation *in vitro*, 293T cells were incubated in the Buffer A (250 mM sucrose, 10 mM HEPES–KOH, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, protease inhibitor cocktail, 50 μg/ml creatine phosphokinase, 10 mM phosphocreatine, 2 mM ATP, 0.1% Triton X-100) for 20 minutes on ice. Cells were then broken with a glass Dounce homogenizer (Jencons). Nuclei were removed by spinning at 790*g* for 10 min at 4 °C. The post-nuclear supernatant was used as cellular extract for Bax degradation assay. The protein extract (in 5 mg/ml protein concentration) was incubated with 2 μg/ml ubiquitin (Sigma) in the Buffer A at 37 °C for up to 5 h. For examining Bax degradation at the whole cell levels, cells were treated with 100 μg/ml cycloheximide (CHX), a protein synthesis inhibitor (Sigma), in the presence or absent of 5 μM MG-132, a proteasome inhibitor (Biomol International) at 37 °C for up to 5 h. Bax protein expression was determined by Western blotting [8].

EGFP-BDS ubiquitination and immunoprecipitation. 297T cells were co-transfected with EGFP-BDS-1 or EGFP-BDS-7 constructs and the HA-tagged ubiquitin expression construct MT123 [19] for 18 h. The transfectants were then treated with 5 μM MG-132 for 5 h. About 1000 μg total proteins in 300 μl were mixed with 20 μl Dynal magnetic beads coated with goat anti-mouse IgG (Dynal) and 2 μg living color anti-EGFP mouse monoclonal antibody, JL-8 (BD, Clontech), anti-EGFP monoclonal antibody. The immunoprecipitation was performed overnight on a rotor 4 °C. The beads coated with HA-ubiquitin-conjugated EGFP-BDS were washed for three times with the lysis buffer and was eluted with a loading buffer and then detected by Western blotting using an anti-HA antibody (Sigma).

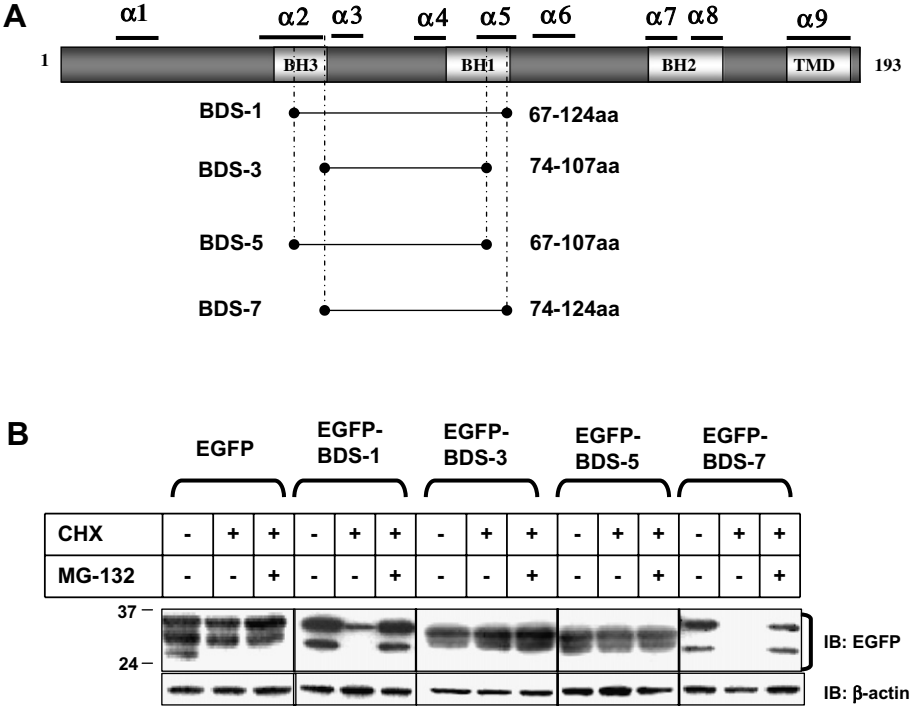

Fig. 2. Screening the Bax degradation sensitive domain. (A) A schematic representation of Bax is shown by the horizontal bar with its four functional domains indicated inside the bar and nine α-helices are shown on the top of the bar. Four deduced Bax degradation sensitive domains, BDS-1, BDS-3, BDS-5, and BDS-7 were listed under the bar. (B) Transfected 293T cells were treated with 100 μg/ml CHX alone or with 5 μM MG-132 for 5 h. The anti-EGFP monoclonal antibody was used at 1:1000 dilution and the anti-β-actin antibody was used at 1:5000 dilution.

Apoptosis measurement by flow cytometry. After transfection for 18 h, cells were washed with PBS. For measuring mitochondrial membrane potential ($\Delta\Psi_m$), cells were incubated with 20 nM Tetramethylrhodamine methylester (TMRM, Molecular Probes) for 15 min at 37 °C. $\Delta\Psi_m$ was determined by the intensity of TMRM by BD LSR II flow cytometer analyzer (BD, Oxford UK). The percentage of $\Delta\Psi_m$ reduction was calculated by the amount of $\Delta\Psi_m^{LOW}$/EGFP$^+$ cells divided by total EGFP$^+$ cells. To determine DNA fragmentation, 293T cells were fixed/permeabilized with 70% ethanol for 30 min, washed with PBS and then stained with propidium iodide (PI, Sigma) for 40 min at 37 °C. PI fluorescence of nuclei was measured by flow cytometer.

Results and discussion

Cells of the different branches of the kingdom of life have developed more or less complex system for special recognition and degradation of proteins with a great variety of destruction signals. The specificity of these systems ascertains that only proteins with destruction signals are rapidly degraded while proteins devoid of such signals are not [20]. This article demonstrates that exposure of Bax α5 helix may serve as a destruction signal for its proteasome-dependent degradation.

Bax is a short-lived protein in 293T cells

Bax degradation activity in the 293T cell line was first tested in a cell-free system. When the post-nuclear fractions were incubated in the degradation buffer containing ubiquitin and Triton X-100, Bax levels declined significantly with time. The half-life of Bax protein in the 293T cells was about 2 h. In comparison, Bcl-2 levels were kept unchanged (Fig. 1A). Bax degradation activity was also determined when the intact 293T cells were treated with CHX

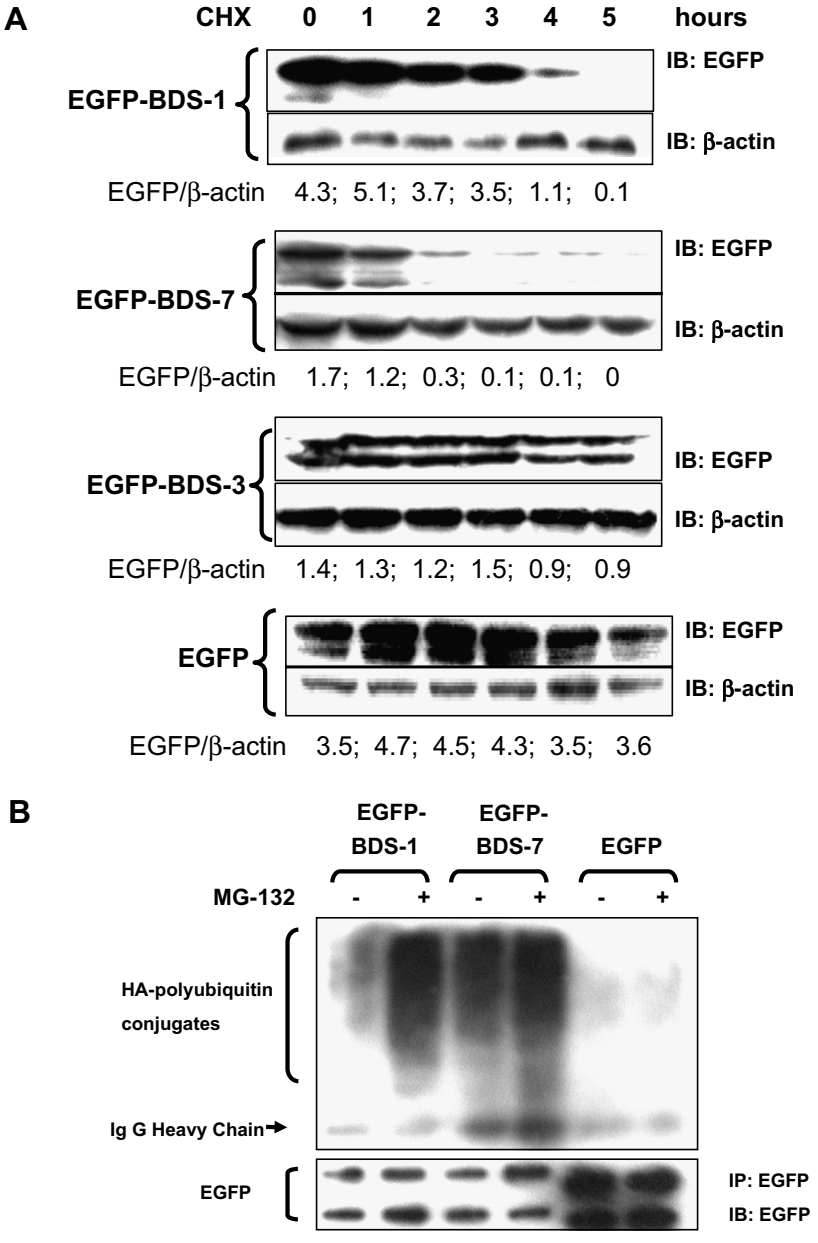

Fig. 3. Lifespan and ubiquitination of BDS-1 and BDS-7. (A) Lifespan. EGFP-BDS-1, EGFP-BDS-7, EGFP-BDS-3, or EGFP transfected 293T cells were treated with 100 μg/ml CHX for up to 5 h. EGFP fusion protein expression was detected by Western blotting. (B) Ubiquitin-conjugation by immunoprecipitation. 293T cells were co-transfected with EGFP-BDS-1, EGFP-BDS-7, or EGFP vector (0.5 μg) and HA-ubiquitin (0.5 μg) for 18 h. The ubiquitin-conjugation with EGFP-fusion protein was detected with the anti-HA antibody (the top panel), and the loading control was shown by probing with EGFP antibody in the Western blotting. IP means immunoprecipitation.

for 5 h. The half-life of the native Bax protein in 293T cells is about 2 h. Again, the native Bcl-2 protein is relatively stable (Fig. 1B). This indicates that Bax, but not Bcl-2, is a short-lived protein in the 293T cell line.

Bax degradation sensitive domain localizes in the α5 helix region

Previous study showed that Bax degradation occurs at the mitochondrial level which can be prevented by tBid. Proteasome inhibitors also have the ability to increase or maintain Bax protein levels—which are critical for Bax activation [8]. We therefore aimed to determine whether Bax degradation sensitive domain localizes in the center of Bax protein which can be exposed after the translocation to mitochondria. Four different lengths of deduced Bax degradation sensitive (BDS) segments, BDS-1 (67–124 aa), BDS-3 (74–107 aa), BDS-5 (67–107 aa), and BDS-7 (74–124 aa), which all share similar sequence with tBid, were fused to the C-terminus of the EGFP-C1 vector (Fig. 2A). The sensitivity to degradation was tested after these constructs were transfected into 293T cells. The expression of EGFP in the EGFP transfectant was not affected by the treatment with CHX, in the presence or absence of MG-132. EGFP-BDS-1 (α2–α5) expression was reduced by CHX but the degradation was prevented by MG-132. The BDS-3 and BDS-5 segments (without the α5 helix) were relatively stable. EGFP-BDS-7 which contains α3, α4, and α5 helices but not the BH3 domain (α2 helix) showed more sensitivity to degradation (Fig. 2B). This suggests that the α5 helix is an essential domain for Bax degradation.

To further confirm the instability of BDS-1 and BDS-7, transfected 293T cells were treated with CHX for 5 h. The half-lives of EGFP-BDS-1 and EGFP-BDS-7 were compared with those of EGFP-BDS-3 and EGFP-vector. The levels of EGFP-BDS-1 and EGFP-BDS-7 almost disappeared after 5 h treatment, indicating about 2.5 h half-lives (Fig. 3A). The EGFP-BDS-3 transfectant showed stable expression. To determine whether BDS-1 or BDS-7 could conjugate with ubiquitin, 293T cells were co-transfected with EGFP-BDS-1, EGFP-BDS-7, or the EGFP vector, together with the ubiquitin-HA construct. Both EGFP-BDS-1 and EGFP-BDS-7 were sensitive to ubiquitin-conjugation in the presence of MG-132 (Fig. 3B). EGFP alone did not show conjugation with ubiquitin in response to MG-132. These results suggest that both BDS-1 and BDS-7 which contain the α5 helix have the Bax ubiquitin-conjugation motif, whereas BDS-3 and BDS-5 without the α5 helix could not conjugate with ubiquitin.

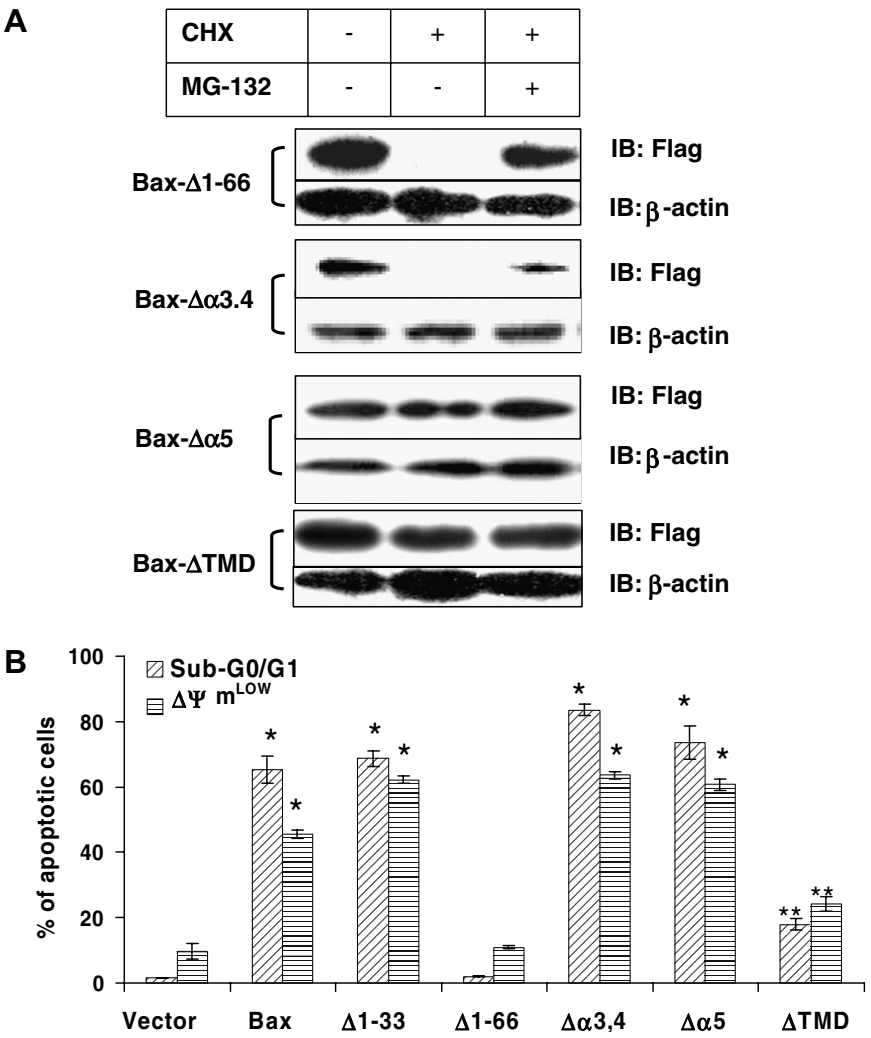

Fig. 4. The sensitivity of Bax deletion mutants to degradation and apoptosis. (A) Bax degradation. 293T cells were transfected with Flag-Bax-Δ1–66, Flag -Bax-Δα3,4 and Flag-Bax-Δα5, Flag-Bax-Δ1–33 (p18 Bax) or Flag-Bax-ΔTMD constructs. After 18 h of transfection, cells were treated with CHX ± MG-132. Expression of Flag-tagged proteins in transfected cells was determined by Western blotting with the anti-Flag antibody (1:1000 dilution). (B) Statistical analysis of apoptosis and reduction in $\Delta\Psi_m$. Values and error bars represent the average and standard derivation of three independent experiments. $^{*}P < 0.001$ and $^{**}P < 0.05$ indicate significantly increased apoptotic events compared with the cells transfected with empty pCMV-Flag vector.

It was reported that the BH3-only protein Bid is sensitive to ubiquitin/proteasome-dependent degradation after being cleaved caspase-8 [18]. The formation of tBid leads two actions, either forming complexes with other members of the Bcl-2 family or being degraded by proteasome, therefore the apoptotic process may be either stimulated or inhibited. BDS-1 and BDS-7 segments containing α5 were quickly degraded when protein synthesis was inhibited by CHX and both were sensitive to ubiquitin-conjugation. In addition, both BDS-1 and BDS-7 share a similar sequence (from α3–α5 helices) with tBid which is sensitive to degradation, suggesting that the α5 helix contains a degradation-sensitive domain. The α5 helix is one of the mitochondrial transmembrane domains of Bax. A hairpin pair of amphipathic helices of α5–α6 has been predicted to form a pore and to be involved in the membrane insertion [21]. Our other study found that the membrane insertion of the α5 helix, which requires additional amino acid residues in the loop between the α5 and α6 helices, can stabilize Bax protein (manuscript in preparation).

Deletion of the α5 helix prevents Bax degradation

Five Bax deletion mutants, Bax-Δ1–66, Bax-Δα3,4, Bax-Δα5, Bax-Δ1–33, and Bax-ΔTMD were constructed within the pCMV-tag-2B vector to further determine their sensitivities to degradation (Supplementary Fig. 1). These constructs were transfected into 293T cells individually and the transfectants were then treated with CHX ± MG-132. Bax-Δ1–66 or Bax-Δα3,4 transfected cells showed a greater susceptibility to degradation when treated with CHX alone. It was also noted that inhibition of proteasome by MG-132 retained their stability. The expression of Bax-Δ1–33 was not detected, probably due to a rapid degradation. BaxΔ1–33 and BaxΔ1–66 with a deletion of the N-terminal α1 helix were sensitive to degradation, suggesting that the α1 helix is important for protecting the α5 helix from exposure. Importantly, the Bax-Δα5 mutant was resistant to degradation (Fig. 4A). This suggests that the α5 helix is the major degradation sensitive domain in the Bax protein. However, deletion of TMD did not destabilize Bax, suggesting that the α5 helix is protected by the N-terminus of Bax. A recent study showed that the C-terminus of Bax is involved in the stabilization of Bax oligomers rather than mitochondrial localization [17]. This indicates that the mitochondrial Bax is sensitive to degradation prior to its membrane insertion or oligomerization with itself or other members.

Deletion of the α5 helix does not prevent Bax-induced apoptosis

The pro-apoptotic activity of these Bax mutants was determined by flow cytometry analysis on both DNA content and $\Delta\Psi_m$. Cells in the sub-G0/G1 and with low $\Delta\Psi_m$ populations ($\Delta\Psi_m^{LOW}$) were defined as apoptotic cells (Supplementary Fig. 2). Similar to the full-length Bax, both Bax-Δα3,4 and Bax-Δα5 constructs induced a significant apoptotic cell death in 293T cells after transfection (Fig. 4B and Supplementary Fig. 2), indicating the α3, α4, or α5 helix alone was not crucial for the pro-apoptosis activity of Bax. This suggests apart from the α5 helix there is at least another important domain within Bax contributing to induce apoptosis. The BH3 domain is a death domain of Bax-inducing apoptosis via oligomerization with Bcl-2 or Bcl-XL. Deletion of the α1 helix and partial BH3 domain (Bax-Δ1–66) abolished the mitochondrial targeting and the pro-apoptosis activity. It was reported that a three-helix unit, comprised of the BH3 (α2 helix) and BH1 domains (α4 helix and α5 helix), as Bax homo-oligomerization domain, is responsible for the apoptotic activity of Bax [13]. Our results demonstrate that detection of the α4 or the α5 helix individually did not affect the apoptotic activity of Bax. However, deletion of C-terminal TMD of Bax led to a significant inhibition of apoptosis.

This study we confirm earlier work [22] that Bax-Δ1–33 (Bax p18) is highly instable. It triggers apoptosis rapidly (Fig. 4B) but the p18 Bax protein degraded quickly even in the absence of CHX (result not shown). This also further confirms that the deletion of the α1 helix can activate Bax by unfolding this molecule and increases both apoptotic and degradation activities. The Bax transmembrane domain (TMD) was neither sensitive to degradation nor able to induce apoptosis (results not shown). Bax-ΔTMD only showed a weak apoptosis inducing ability (Fig. 4B) but, it was insensitive to CHX-triggered degradation (Fig. 4A).

In summary, Bax is a short-lived protein and it turns over rapidly in cancer cells. The Bax α5 helix contains an ubiquitin-sensitive domain and the exposure of the α5 helix could serve as a destructive signal. Our other study demonstrates that stable insertion of the α5 helix can prevent its degradation. We propose that Bax translocation and its conformational change trigger two opposite actions, i.e. membrane insertion and degradation. It remains to be explored as how the dual roles of the α5 helix are regulated.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.03.122.

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