**Subtilase cytotoxin induces apoptosis in HeLa cells by mitochondrial permeabilization
via activation of Bax/Bak, independent of C/EBF-homologue protein (CHOP), Ire1 α or
JNK signaling**

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Abstract

Subtilase cytotoxin (SubAB) is an AB₅ cytotoxin produced by some strains of Shiga-toxigenic *Escherichia coli*. The A subunit is a subtilase-like serine protease and cleaves an endoplasmic reticulum (ER) chaperone, BiP, leading to transient inhibition of protein synthesis and cell cycle arrest at G₁ phase, and inducing caspase-dependent apoptosis via mitochondrial membrane damage in Vero cells. Here we investigated the mechanism of mitochondrial permeabilization in HeLa cells. SubAB-induced cytochrome *c* release into cytosol did not depend on mitochondrial permeability transition pore (PTP), since cyclosporine A did not suppress cytochrome *c* release. SubAB did not change the expression of anti-apoptotic Bcl-2 or Bcl-XL and pro-apoptotic Bax or Bak, but triggered Bax and Bak conformational changes and association of Bax with Bak. Silencing using siRNA of both *bax* and *bak* genes, but not *bax*, *bak*, or *bim* alone, resulted in reduction of cytochrome *c* release, caspase-3 activation, DNA ladder formation and cytotoxicity, indicating that Bax and Bak were involved in apoptosis. SubAB activated ER transmembrane transducers, Ire1 α , and cJun N-terminal kinase (JNK), and induced C/EBF-homologue protein (CHOP). To investigate whether these signals were involved in cytochrome *c* release by Bax activation, we silenced *ire1 α* , *jnk* or *chop*; however, silencing did not decrease SubAB-induced cytochrome *c* release, suggesting that these signals were not necessary for SubAB-induced mitochondrial permeabilization by Bax activation.

Keywords; Subtilase cytotoxin, ER stress, apoptosis, Bax, Bak,

1. Introduction

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3 A new member of the AB₅ toxin family, named subtilase cytotoxin (SubAB), was identified
4 in *E. coli* O113:H21 strain 98NK2, which produced Stx2 and was responsible for an outbreak of
5 hemolytic uremic syndrome (HUS) [1]. SubAB A subunit, a 35 kDa protein, shares sequence
6 homology with a subtilase-like serine protease of *Bacillus anthracis* and the toxin was named
7 ‘subtilase cytotoxin’. The A subunit cleaves at a specific single site of ER chaperone BiP[2]. B
8 subunits bind with high specificity to *N*-glycosylated membrane proteins [3] and in particular,
9 glycans terminating in the sialic acid *N*-glycolylneuraminic acid [4]. $\alpha 2\beta 1$ integrin has been
10 shown to be one of the receptors responsible for vacuolating activity of B subunits [3].

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12 SubAB is lethal for mice, causing extensive microvascular thrombosis as well as necrosis in
13 the brain, kidney and liver, and apoptosis in the spleen, kidney and liver [5]. These findings are
14 similar to the histopathologic, biochemical and hematologic changes seen in human HUS.
15 Mutagenesis of a critical active site Ser residue in SubA abolished the toxicity, indicating that
16 BiP cleavage is central to the mechanism of action of SubAB. SubAB cytotoxicity of Vero and
17 HeLa cells correlated with A subunit enzymatic activity[1, 6].

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19 BiP, a target of SubAB, localizes mainly in the ER lumen. ER is the site for synthesis, folding,
20 modification and trafficking of secreted and cell-surface proteins. BiP is a master regulator of
21 ER function and homeostasis [7]. Cleavage of BiP by SubA induces ER stress, which was
22 demonstrated by activation of double-stranded RNA-activated protein kinase-like ER kinase
23 (PERK) and eukaryotic initiation factor-2 α (eIF2 α), leading to transient inhibition of protein
24 synthesis, and induction of CHOP, with cell cycle arrest in G₁ phase as a result of
25 down-regulation of cyclin D1 [8]. The other membrane stress sensors, ATF6 and Ire1 α , were
26 also activated by SubAB [9]. To avoid cell death, ER chaperone proteins eliminate the stress;
27 when this fails, cell death occurs. In SubAB-treated cells, BiP, a chaperone protein, was
28 cleaved and inactivated, resulting in continuous ER stress, thus leading to cell death. Lass et al.
29 [10] explored the mechanism of ER stress induced by SubAB in human cells lines, focusing on
30 its effects on ER-associated degradation. In a previous report, we showed that in Vero cells
31 SubAB induced caspase-dependent apoptosis via mitochondrial membrane damage [11]. We
32 did not clarify the mechanism by which signal SubAB induced mitochondrial permeabilization
33 and apoptosis.

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35 Bcl-2 family member proteins strictly control cell death in response to diverse stimuli, e. g.,
36 DNA damage, viral infection, growth factor deprivation [12, 13]. The family members are
37 grouped into two classes. One class (pro-survival proteins) inhibits apoptosis, whereas a second
38 class (pro-apoptotic proteins), which promotes apoptosis, includes the death mediators, Bax
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3 and Bak, and other proteins. These proteins possess a conserved BH-3 domain, which can bind
4 and regulate the pro-survival Bcl-2 proteins and promote apoptosis. In this report, we show in
5 HeLa cells that mitochondrial cytochrome *c* release was dependent on Bax/Bak activation. We
6 further report that signaling from CHOP, Ire1 α , or JNK, which were activated by
7 SubAB-induced BiP cleavage, did not suppress cytochrome *c* release by Bax activation,
8 although those mediators were involved in ER-stress induced apoptosis in other cell types using
9 different death stimuli [14, 15].
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18 **2. Results and Discussion**

20 **2.1. SubAB induces apoptosis in HeLa cells by intrinsic pathway via mitochondria.**

21 SubAB induces apoptotic cell death of HeLa cells, similar to that seen with Vero cells [11]. The
22 50% inhibitory dose in HeLa cells was, however, ~50 ng/ml, which was ~100 times greater than
23 that needed with Vero cells. 100~200 ng/ml of SubAB induced activation of caspases-3, -8, and
24 -9 and PARP cleavage (Fig. 1A). As we previously reported, SubAB-induced apoptosis was
25 dependent on BiP cleavage, which was occurred within 60 min (Fig. 1B); the catalytically
26 inactive mutant, SubAB(S272A), did not cleave BiP (Fig. 1B) and did not induce apoptosis.
27 SubAB-induced apoptosis resulted from activation of the intrinsic pathway in which
28 cytochrome *c* release from mitochondria triggers the formation of the apoptosome composed of
29 Apaf-1 and procaspase-9. Activated caspase-9 then stimulated activation of caspase-3. General
30 caspase inhibitor Z-VAD-FMK (VAD) suppressed apoptosis, with reduction in Annexin-V
31 binding [11]. VAD and inhibitors specific to caspase-3, 8 and 9, however, did not suppress
32 cytochrome *c* release in HeLa cells (Fig.1C), suggesting that not only cytochrome *c* release but
33 also caspase activation is critical for SubAB-induced cell damage by apoptosis. It may also
34 suggest that cytochrome *c* release by SubAB may occur upstream of caspase activation.
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47 **2.2. SubAB induces mitochondrial membrane damage in a Bax/Bak-dependent manner**

48 Permeabilization of mitochondria outer membrane (OMM) can be achieved by several different
49 mechanisms, including pore formation by pro-apoptotic Bcl-2 family proteins. We first investigated
50 the levels of Bcl-2 family proteins (Fig. 2). Expression levels of pro-survival family members, Bcl-2
51 and Bcl-XL, were not changed by incubation with SubAB for up to 30 h. Mcl-1 in contrast was
52 decreased. SubAB(S272A) did not induce Mcl-1 decrease. Mcl-1 is primarily localized to the outer
53 mitochondrial membrane and promotes cell survival by suppressing cytochrome *c* release from
54 mitochondria via heterodimerization with and neutralization of pro-apoptotic Bcl-2 family members
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1 including Bak [16, 17]. Therefore the decrease in Mcl-1 may be a factor contributing to induction of
2 apoptosis. We investigated the effect of Mcl-1 overexpression (Supplementary Figure S1). In cells
3 overexpressing Mcl-1, slight suppression of cytochrome *c* release was observed but it was not
4 significant. Caspase-3 level was not suppressed. Levels of cell death mediators, Bax and Bak, were not
5 different (Fig. 2). These data suggested that SubAB-induced cell death was not caused by increased
6 amounts of pro-apoptotic proteins, Bax and Bak, or by reduction of pro-survival proteins, Bcl-2 and
7 Bcl-XL. The level of a BH3-only protein, Bim, was not increased, however, the protein was
8 dephosphorylated in a time-dependent manner, which was determined by its faster mobility on
9 SDS-PAGE (Fig. 2). SubAB(S272A) did not induce the dephosphorylation of Bim. A role for Bim in
10 apoptosis was proposed recently [18-20] ; it has been suggested that ER stress increases
11 dephosphorylated Bim, which inhibits Bcl-2, a pro-survival protein, leading to Bax/Bak activation and
12 cell death [21]. To confirm whether SubAB toxicity follows those pathways, we investigated whether
13 in cells with knockdown of Bim using Bim siRNA, cytochrome *c* release by SubAB was suppressed.
14 We observed, however, that cytochrome *c* release was increased (Supplementary Figure S2). These
15 data suggested that Bim alone was not responsible for induce SubAB-induced mitochondrial damage.
16 SubAB but not mutant SubAB(S272A) decreased another regulator, Bad, which was found to induce
17 apoptosis by suppressing Bcl-XL activity [22] or by dephosphorylation of Bad which sensitizes the
18 mitochondrial permeability transition pore (PTP) to Ca^{2+} , independent of Bax or Bak, through a
19 Bcl-xL-dependent process [23]. Bad overexpression induces apoptosis in JEG-3 cells [24]. Therefore,
20 decrease in both phosphorylated- and dephosphorylated-Bad (Fig. 2) may inhibit cell death following
21 SubAB treatment. Therefore, involvement of Bad in SubAB-induced apoptosis seems unlikely.

22 Interaction of pro-apoptotic Bcl-2 family members with components of the PTP, which
23 consists of voltage-dependent anion channel, the adenine nucleotide translocator, and
24 cyclophilin D, results in mitochondrial depolarization and swelling, followed by mitochondrial
25 outer membrane rupture and release of the inner membrane content [23]. This PTP formation
26 can be prevented by cyclosporine A [25, 26]. In our system, cyclosporine A enhanced
27 SubAB-induced cytochrome *c* release (Fig. 3), suggesting that SubAB-induced mitochondrial
28 permeabilization did not depend on PTP.

29 Total Bax and Bak content did not change by SubAB-treatment (Fig. 2). It is known,
30 however, that in response to apoptotic stimuli, Bax translocates from cytosol to mitochondria
31 [27], and integrates into the OMM as mono- or hetero oligomers with Bak, leading to pore
32 formation [28-30]. We examined whether subcellular localization of Bax and Bak was changed
33 by SubAB. Bax was found, however, in both cytosolic and membrane fractions in untreated

control cells and no increase of Bax in the membrane fraction, which contains mitochondria, was observed following treatment with SubAB. Thus, in HeLa cells, Bax may be localized not only in cytosol but also in mitochondria or other membranes. In contrast, Bak was found only in membrane fractions (data not shown). SubAB, however, induced Bax conformational changes in a time-dependent manner, by flow cytometric analysis using conformation-specific Bax antibody (clone 3) (Fig. 4A). In contrast, HeLa cells incubated without toxin did not show any conformational change of Bax (data not shown). We further investigated Bax and Bak association in SubAB-treated cells using immunoprecipitation with conformation-specific Bax antibody (Fig. 4B). Conformationally changed Bax was only found in SubAB-treated cells but not in control without toxin or SubAB(S272A)-treated cells; Bak was precipitated by antibody against conformation-specific Bax, suggesting complex formation of Bax and Bak (Fig. 4B, middle blot). Conformation-specific Bak was clearly increased in SubAB-treated cells compared to control or SubAB(S272A)-treated cells (Fig. 4B, lower blot). To evaluate a Bax effect on cytochrome *c* release, we knocked down Bax with siRNA. In cells in which Bax level was decreased, however, no suppression of cytochrome *c* release was observed (Fig. 5 and Supplementary Figure S2). In contrast, in cells in which both Bax and Bak were knocked down (Fig. Supplementary Figure S3), cytochrome *c* release and activation of caspase-3 by SubAB were significantly suppressed (Fig. 5A); in agreement with this result, SubAB-induced DNA fragmentation and SubAB cytotoxicity were also suppressed (Fig. 5B and C).

2.3. Pathway to induce Bax/Bak activation after BiP cleavage

Above data showed that Bax/Bak were involved in SubAB-induced cytochrome *c* release. However, how Bax/Bak were activated was not known. Several pathways have been implicated in ER stress-induced apoptosis [14]. A transcription factor, CHOP, which is induced by ER stress at the transcript level, was reported to induce apoptosis mediated by downregulation of Bcl-2 [31], by upregulation of Bim [21], and by translocation of Bax from cytosol to mitochondria [32]. Apoptosis induction by CHOP was demonstrated with CHOP^{-/-} mice [33]. In HeLa cells, SubAB caused CHOP induction in a time-dependent manner (Fig. 6A) similar to a previous report [10]. To investigate whether CHOP activation was correlated with SubAB-induced apoptosis, *chop* was silenced with siRNA. In cells in which CHOP induction by SubAB was suppressed (Fig. 7A), SubAB-induced cytochrome *c* release at 30 h was not suppressed but rather increased (not significant) but no difference was observed at 34 h (Fig. 7B). The other is a recently reported pathway in which p53 activated BH-3-only protein PUMA and NOXA at the transcript level [34], however, PUMA was slightly decreased by SubAB

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3 treatment (Fig. 2) and p53 level was not increased (data not shown), suggesting that increased
4 PUMA expression by p53 was not the cause of SubAB-induced apoptosis.
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6 JNK plays a critical role in intrinsic apoptotic pathways. It activates apoptotic signaling by
7 upregulation of pro-apoptotic genes through the activation of specific transcription factors or by
8 directly modulating the activities of mitochondrial pro- and anti-apoptotic proteins through
9 phosphorylation (e. g., Bim, Bmf, Bcl-2) [15, 18]. Other studies show that in ER stress, JNK
10 activation, coupled with Ire1 α -TRAF2-ASK1 signaling, induced apoptotic cell death [35, 36].
11 SubAB treatment of HeLa cells induced rapid phosphorylation of JNK at around 90 min and
12 continuing for up to 4 h, with subsequent decrease (Fig. 6B and 9B) similar to previous report
13 [10]. We found that JNK level was decreased by SubAB (Fig. 6B). In contrast, SubAB
14 significantly increased Ire1 α level after 6 h and Ire1 α was activated by phosphorylation, which
15 was detected by slower migration by SDS-PAGE (Fig. 6C). Phosphorylation was found after ~3
16 h and continued up to 30 h. We asked whether Ire1 α knockdown would suppress JNK
17 activation and apoptosis. Even in cells in which Ire1 α transcript level was almost completely
18 knocked down, however, JNK phosphorylation was not suppressed (Fig. 8A). It seems that JNK
19 activation was not a downstream event following Ire1 α activation. Knockdown of Ire1 α did not
20 inhibit, rather it significantly increased cytochrome *c* release, caspase-3 activation and PARP
21 cleavage, indicating that this SubAB-induced apoptotic signal pathway did not follow a
22 pathway involving ER stress sensor Ire1 α activation (Fig. 8B). Ire1 α signaling might enhance
23 cell survival. Next, we investigated the effect of gene silencing of JNK1 (Fig. 9). In cells in
24 which JNK1 level was decreased (Fig. 9A), SubAB-induced phosphorylation of JNK1 was
25 suppressed (Fig. 9B), however, cytochrome *c* release and caspase-3 activation were increased
26 (Fig. 9C). These data suggested that JNK signaling pathway following treatment with SubAB
27 was an anti-apoptotic signal. A role of JNK activation in pro- and anti-apoptotic functions
28 depends on cell type, nature of the death stimulus [15]. A recent study revealed that JNK can
29 suppress apoptosis via phosphorylation of the proapoptotic Bcl-2 family protein, Bad [37].
30 Since SubAB did not increase Bad phosphorylation (Fig. 2), anti-apoptotic function of JNK by
31 SubAB may not be caused by Bad phosphorylation, We tried to confirm this anti-apoptotic
32 effect of JNK by investigating whether overexpression of JNK protected cells from
33 SubAB-induced apoptosis however, following a ~2 fold increase of JNK, no difference in
34 cytochrome *c* release was found (data not shown).
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57 SubAB treatment induces prolonged ER stress detected by continuous BiP cleavage. In
58 SubAB-treated cells, stress sensor Ire1 α expression was significantly increased and activated
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from 3 h up to 30 h (Fig. 6C). In contrast, PERK signaling was induced very quickly, as shown in Vero cells by eIF2 α phosphorylation, and attenuated at 3 h [8]. Similarly, in HeLa cells, eIF2 α phosphorylation was detected only for short periods (data not shown). Signaling through Ire1 α and PERK by SubAB are opposite from the data reported in [38]. In their case, Ire1 α signaling was attenuated during prolonged ER stress and in contrast, ATF6 and PERK signaling persisted. They suggested that Ire1 α attenuation with CHOP activation causes cell death. In our case, prolonged Ire1 α activation as well as continuous CHOP activation were detected. However, CHOP knockdown did not significantly change mitochondrial membrane damage and Ire1 α knockdown increased apoptotic signals, suggesting that Ire1 α or CHOP may inhibit cell death following SubAB treatment.

In this paper, we demonstrated that SubAB-induced cytochrome *c* release was mainly regulated by Bax and Bak. Those proteins might have a redundant function involved in the regulation of SubAB-induced apoptosis, because only in cells where both genes were knocked down was apoptosis suppressed. SubAB induced conformational changes of Bax and Bak, with complex formation perhaps leading to stable pores, which promote cytochrome *c* release [30]. It appears that complex formation is not always necessary to induce cytochrome *c* release, because knockdown of one gene did not decrease cytochrome *c* release. There are also similar reports that both genes knockdown are necessary to suppress cytochrome *c* release [39]. The activation pathway for Bax and Bak has not been determined. In mouse cells, ER stress can trigger cellular apoptosis through the activation of caspase-12, which resides on the outside of ER membrane, and is cleaved and activated during ER stress [14]. In human cells, caspase-12 is not expressed; existence of alternative proteins in the ER membrane would be expected.

SubAB-induced cytotoxic effects can be summarized as follows. SubAB is bound to sensitive cells (e.g., Vero cells, HeLa cells), internalized by endocytosis, and transported from the Golgi apparatus to ER in a retrograde manner. The pathway is illustrated in Fig. 10. In the ER lumen, SubAB cleaves BiP, leading to ER stress. SubAB-induced ER stress signals (e.g., Ire1 α , CHOP, JNK1) are not involved in cytochrome *c* release, and might rather promote cell survival. ER stress signals are transferred to Bax/Bak leading to a Bax-Bak complex or maybe mono oligomers of Bax or Bak and cytochrome *c* release, which activates caspase-3, leading to cell damage in a caspase-dependent way.

3. Materials and Methods

3.1. Cells and reagents

HeLa cells were cultured at 37°C in a humidified 5% CO₂ atmosphere in Eagle's Minimum Essential Medium (EMEM) containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. General caspase inhibitor (Z-VAD-FMK) was obtained from BD Biosciences Pharmingen, caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) from R&D Systems, and caspase-3 inhibitor (Z-DQMD-FMK) from Calbiochem. Cyclosporine A was obtained from Wako Pure Chemical. Primary antibodies were anti-cytochrome *c* (sc-13560), anti-GAPDH (sc-25778) and anti-CHOP/GADD153(B-3)(sc-7351) (Santa Cruz Biotechnology); anti-caspases-3 (9662), -9 (9502), and -8(9746), anti-cleaved capases-3 (9661) and -9 (9501), anti-PARP (9542) and -cleaved PARP (9541), anti-Bax (2772), anti-Bak (3814S), anti-Bcl-2 (2870), anti-Bcl-XL (2764), anti-Mcl-1(4572), anti-Bad (9292), anti-p-Bad (9296), anti-Bim (2819), anti-Puma (4976) and anti-Ire1 α (3294S) (Cell Signaling); anti-JNK1(633101) (BioLegend); anti-Bax (clone 3) (610982), anti-BiP (610978) and anti-p-JNK (612540) (BD Biosciences); anti-p53 (Ab-6) (OP-43-100UG) (Oncogene); anti-Bak (Ab-2) (AM04) (Calbiochem). siRNA for Bim, Bax, Bak, CHOP/GADD153, and negative controls were from Santa Cruz Biotechnology. siRNA for JNK1 was from Invitrogen, and that for Ire1 α was from Qiagen.

3.2. Preparation of SubAB and SubAB(S272A)

Recombinant His-tagged SubAB and the catalytically inactive mutant, SubAB(S272A), were purified by Ni-NTR chromatography as previously reported [6].

3.3. Detection of cytochrome c release from mitochondria

To evaluate cytochrome *c* release from mitochondria into cytosol, cells were treated with SubAB (100 ng/ml) for indicated times, collected with a cell scraper and homogenized for 5 min in buffer (75 mM KCl, 1 mM Na₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose, 1 mM EDTA) containing 50 μ g/ml digitonin and protease inhibitor cocktail (Roche Diagnostics). Following centrifugation at 10,000 x g for 10 min, the supernatant was collected as cytosolic fraction and cytochrome *c* was detected by Western blotting.

3.4. Gene silencing in HeLa cells

HeLa cells (1 x 10⁵ cells) in a 12 well-plate were cultured overnight (50-60% confluent) and were transfected with 10~30 pmol negative control (NC) siRNA or siRNA for Bax, Bak, Bim, JNK1, CHOP, or Ire1 α in Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen) transfection reagent for 48 h following the company's instructions. Transfection efficiency and effect were evaluated by Western blotting using each antibody.

3.5. Western blotting analysis

Cells were plated in a 12-well dish at 3×10^5 per well one day prior to treatment. After treatment, cells were washed with PBS, lysed with SDS-sample buffer [0.0625M Tris, (pH 6.8), 1% SDS, 10% glycerol, 2.5% mercaptoethanol, 0.001% bromophenol blue] and heated at 100°C for 5 min before proteins were analyzed by SDS-PAGE. After electrophoresis at room temperature, separated proteins were transferred onto PVDF membranes at 100 V for 1 h. Membranes were blocked with 5 % non-fat milk in TTBS (20 mM Tris (pH 7.6), 137 mM NaCl, 0.1% Tween 20) for 30 min and then incubated with primary antibodies for 1 h at room temperature or sometimes overnight at 4°C. After washing the membranes three times for 5 min with TTBS, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. Bands were visualized using Las 1000 (Fuji film). To investigate the effect of caspase inhibitors, cells were treated with inhibitors for 30 min prior to treatment with toxin and then incubated for 30 h with SubAB.

3.6. Intracellular flow cytometric analysis

Assay was performed by the method of Dewton et al. [28]. Briefly, cells were fixed with 2% formaldehyde for 10 min at room temperature, then washed in PBS and incubated for 45 min on ice in the presence of anti-Bax clone 3 monoclonal antibody (1 µg/ml) diluted in permeabilization buffer (1.25% BSA, 0.1% saponin in PBS). As a negative control cells were incubated in the absence of primary antibody. Cells were washed with 0.5% BSA/PBS and incubated with Alexa 488 conjugated secondary antibody (1:100). Cells were washed with 0.5% BSA/PBS, resuspended in PBS and analyzed by FACSCantoII.

3.7. Construct of Mcl-1 expression plasmid and transfection

Total RNA was extracted from HeLa cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 5 µg of total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). Primers used for Mcl-1 amplification were 5'-caccatgtttggcctcaaaagaacgcggt-3' and 5'-tcttattagatatgcaaaccagctctac-3'. cDNA was amplified in 50 µl of PrimeSTARTM GC PCR mixture according to the manufacturer's protocol (TaKaRa Bio). The PCR conditions were as follows: 30 cycles of 98 °C for 10 s and 68 °C for 90 s. To add 3' adenine-overhangs, ExTaq polymerase (0.5 µl, TaKaRa Bio) was incubated with the reaction mixture at 72 °C for 10 min. PCR products were subjected to electrophoresis on 1% agarose gels containing ethidium bromide, and the band was extracted by Gel Extraction Kit (Qiagen) and then inserted in the cloning site of pcDNA3.2/V5/GW/D-TOPO vector (Invitrogen). The Mcl-1 gene was confirmed by sequencing. Cells were cultured in a 12 well dishes (1×10^5 /well) overnight and

transfected with 1 µg of plasmids using lipofectamine LTX and Plus reagent (Invitrogen). After 24 h incubation, cells were treated with SubAB for 30 h.

3.8. Immunoprecipitation

Immunoprecipitation of Bax/Bak complex was carried out as described previously [40]. Briefly, HeLa cells (3×10^5 / 6 cm plate) were treated with SubAB, SubAB(S272A) or control PBS for 36 hr. After washing with ice-cold PBS, cells were solubilized with lysis buffer (10 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 2% CHAPS, pH 7.4) containing protease inhibitor cocktail (Roche Diagnostics) and incubated for 30 min on ice. After centrifugation at 17,400 x g for 15 min at 4°C, solubilized extracts (150 µg/ 200 µl) were collected and incubated with 0.25 µg of normal mouse IgG (Santa Cruz Biotechnology), anti-Bax antibody (clone 3) (BD Bioscience) or anti-Bak antibody (Ab-2) (Calbiochem) at 4 °C for 2 hr.

Immunoprecipitates were collected by incubation with protein G-Sepharose (Invitrogen) for 1 h, followed by centrifugation for 1 min at 4 °C. After immunocomplexes were washed with lysis buffer three times, proteins were dissolved in SDS-sample buffer, applied to SDS-PAGE in 15% gels, transferred to PVDF membranes, and then analyzed by Western blotting using anti-Bax or anti-Bak antibodies (Cell Signaling)

3.9. Cytotoxicity

Cytotoxicity Detection Kit^{PLUS} [LDH] (Roche Diagnostics) was used to evaluate the cell cytotoxicity according to manufacturer's instructions. Briefly, cells were treated with or without SubAB (200 ng/ml) for the indicated time and then assayed for LDH release in culture medium. To determine the percentage cytotoxicity, the average absorbance values of the triplicate samples and controls were calculated. Cell cytotoxicity was calculated from the values using the following formula: cell cytotoxicity (% of control) = (the absorbance values of SubAB-treated cells minus the absorbance values of untreated cells)/(the absorbance values of high control by cell lysis solution treatment minus the absorbance values of untreated cells) x100.

3.10. Apoptosis

To define the apoptotic cells, we used Cell Death Detection ELISA^{PLUS} ELISA kit (Roche Diagnostics) as described in the manufacturer's instructions. Briefly, cells were treated with SubAB (200 ng/ml), SubAB(S272A) or control PBS for indicated times and lysed with 200 µl of cell lysis buffer. Lysates from floating and adherent cells were added to a streptavidin coated microplate and incubated with a mixture of anti-histone-biotin and anti-DNA peroxidase. After washing the plate with incubation buffer, peroxidase substrates (ABTS) were added to each

well and absorbance was measured at 405 nm. The enrichment factor quantified the specific enrichment of mono- and oligonucleosomes released into the cytoplasm and was calculated using the following formula: enrichment factor = (the absorbance of dying/ dead cells)/(the absorbance of control cells).

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Abbreviations used are: PTP, mitochondrial permeability transition pore; JNK, cJun N-terminal kinase; CHOP, C/EBF-homologue protein; ER, endoplasmic reticulum; HUS, hemolytic uremic syndrome; PERK, double-stranded RNA-activated protein kinase-like ER kinase; eIF2 α , eukaryotic initiation factor-2 α ; EMEM, Eagle's Minimum Essential Medium; OMM, mitochondria outer membrane.

Legends to Figures

Fig. 1. Effects of SubAB in HeLa cells.

A. SubAB-induced caspase activation in HeLa cells. Cells were incubated with 100 ng/ml of SubAB for 30 h, and then detached from the substratum with cell scraper, collected by centrifugation, washed once with PBS, and then lysed with 100 μ l of SDS-sample buffer. Western blotting was performed as described in Materials and Methods. B. Cells were incubated with 100 ng/ml of SubAB or SubAB(S272A) for indicated times, and then washed once with PBS, and then lysed with 100 μ l of SDS-sample buffer. Western blotting was

performed as described in Materials and Methods. C. Cells were incubated with various caspase inhibitors (50 μ M) for 30 min, followed by incubation with SubAB for 30 h. Cytochrome *c* release into the cytoplasmic fraction was determined as described in Materials and Methods. The data shown are representative of three separate experiments. 1; control without inhibitor 2; general caspase inhibitor, 3; caspase-3 inhibitor, 4; caspase-8 inhibitor, 5; caspase-9 inhibitor, 6; control without inhibitor.

Fig. 2. Effects of SubAB on expression of Bcl-2 family proteins.

Cells were plated in a 12-well dish at 3×10^5 per well one day prior to incubation with SubAB or catalytically inactive SubAB(S272A) (200 ng/ml) for indicated times. Cells were then detached from the substratum with cell scraper, collected by centrifugation, washed once with PBS, and then lysed with 100 μ l of SDS-sample buffer. Western blotting was performed as described in Materials and Methods. An upper arrow indicates phosphorylated Bim. Experiments were performed three times; a representative blot is shown.

Fig. 3. Effect of cyclosporine A on SubAB-induced cytochrome *c* release.

Cells were grown overnight, and then cyclosporine A was added at the indicated concentration for 30 min before addition of SubAB (100 ng/ml) and incubation for 30 h. Cytochrome *c* and caspase-3 were quantified by Western blotting. Data are representative of three experiments.

Fig. 4. SubAB-induced Bax activation in HeLa cells. A. FACS analysis of SubAB-induced Bax conformational change. Cells were grown overnight and then incubated with or without SubAB (200 ng/ml) for indicated times. Intracellular flow cytometric analysis was performed as described in Materials and Methods. Control: without first antibody (Bax, clone 3). Graph shows % of conformationally changed Bax (subtracted the amount of control). Data shown are representative of three experiments. B. Conformational changes and association of Bax and Bak. Cells were treated with 200 ng/ml of SubAB, SubAB(S272A), or no toxin for 30 h and then cells were lysed and immunoprecipitated with conformation-specific Bax or Bak antibody as described in Materials and Methods. Left panel shows the total lysate and right panel shows the Bax and Bak, which were immunoprecipitated by conformation-specific anti-Bax or -Bak antibody.

Fig. 5. Effect of Bax or Bak silencing on SubAB-induced cytochrome *c* release from mitochondria.

A. Cells were grown overnight, and silencing of *bax* or *bak* alone or both *bax* and *bak* gene was performed with Bax-, Bak-, or NC-siRNA as described in Materials and Methods. Forty-eight h after transfection, cells were incubated with SubAB (200 ng/ml) for 30 h or 34 h. SubAB-treated cells were analyzed by Western blotting with antibodies against cytochrome *c* or caspase-3. Cytochrome *c* release was determined as described in Materials and Methods. The blots shown are representative of three other experiments. Right panel shows the densitometric analysis of cytochrome *c* release and caspase-3. Data are the means $\pm$ SD value from three separate experiments. * $p < 0.07$, ** $p < 0.05$. B. DNA fragmentation of SubAB-treated cells was analyzed as described in Materials and Methods. Data are the means $\pm$ SD value from three separate experiments. C. Cytotoxicity of SubAB-treated cells was analyzed as described in Materials and Methods. Data are the means $\pm$ SD value from three separate experiments.

Fig. 6. Changes of expression levels of CHOP (A), JNK1 or p-JNK (B) and Ire1 α (C) by incubation with SubAB.

Cells were grown overnight, and then incubated with SubAB (200 ng/ml) for indicated times. After incubation, cells were scraped from the plate, washed once with PBS, solubilized with 1x SDS sample buffer and then heated for 5 min. Samples were analyzed by Western blotting as described in Materials and Methods. Blots shown are representative of at least three experiments.

Fig. 7. Effect of CHOP silencing on SubAB-induced cytochrome *c* release from mitochondria in HeLa cells. Experiments were performed similar to those in the legend to Supplementary Figure S2. A. After transfection of CHOP- or NC-siRNA for 48 h, cells were incubated with SubAB for 30 h and induction of CHOP was quantified by Western blotting. B. Transfected cells were incubated with SubAB for 30 or 34 h and cytochrome *c* release into the cytoplasmic fraction was determined as described in Materials and Methods. Blots shown are representative of three experiments. Quantification of cytochrome *c* was performed by densitometer (lower panel). Data are the means $\pm$ SD value from three separate experiments.

Fig. 8. Effect of Ire1 α silencing on SubAB-induced cytochrome *c* release from mitochondria. Ire1 α silencing was performed as described in Materials and Methods. A. Cells transfected with Ire1 α - or NC-siRNA were incubated with SubAB (200 ng/ml) for indicated times and then analyzed by Western blotting with antibodies against Ire1 α , p-JNK, or GAPDH. A blot representative of three other experiments is shown. B. Cells transfected with Ire1 α - or NC-siRNA were incubated with SubAB (200 ng/ml) for 30 h and then samples for Western blotting were prepared. Blots were analyzed with antibodies against caspase-3, cytochrome *c*, cleaved PARP and GAPDH. The blots shown are representative of three other experiments.

Fig. 9. Effect of JNK silencing on SubAB-induced cytochrome *c* release from mitochondria. JNK1 silencing was performed as described in Materials and Methods. A. Reduction of JNK1 protein level was confirmed by Western blotting with anti-JNK antibodies. B. Cells transfected with NC- or JNK1-siRNA were incubated with SubAB (200 ng/ml) for indicated times and then cells were analyzed by Western blotting with antibodies reactive with p-JNK. C. Cells transfected with NC- or JNK1-siRNA were incubated with SubAB (200 ng/ml) for 30 or 34 h and then samples for Western blotting were prepared. Blots were analyzed with antibodies reactive with caspase-3, cytochrome *c* and GAPDH. The blots shown are representative of three other experiments. Quantification of cytochrome *c* was performed by densitometer (right panel). Data are the means $\pm$ SD value from three separate experiments. *, $p < 0.02$, ** $p < 0.07$.

Fig. 10. Proposed pathway of SubAB-induced apoptosis. See text for additional details.

Supplemental information

Figure legends

Figure S1. Effect of overexpression of Mcl-1 on SubAB-induced cytochrome *c* release. Cells (1×10^5 cells/well) in 12 well plates were grown overnight and transfected with 1 μ g of vector plasmid or V5-tagged Mcl-1 cDNA expression plasmid using lipofectamine LTX and Plus reagent. After incubation for 24 h, cells were treated with or without SubAB (200 ng/ml)

for 30 h, and cytochrome *c* or caspase-3 was detected by Western blotting. Right panel shows quantification of cytochrome *c* released by densitometer. Data are the means $\pm$ SD value from three separate experiments.

Figure S2. Effect of Bim or Bax silencing on SubAB-induced cytochrome *c* release.

Cells were grown overnight, and silencing of *bax* or *bim* gene was performed with Bax- or Bim-siRNA or NC-siRNA as described in Materials and Methods. Forty-eight h after transfection, cells were incubated with SubAB (200 ng/ml) for 30 h. A. Reduction of Bax or Bim protein level was confirmed by Western blotting with anti-Bax or Bim antibodies. Right panel shows quantification of Bax or Bim by densitometer. Data are the means $\pm$ SD value from three separate experiments. B. Cytochrome *c* release into the cytoplasmic fraction was determined as described in Materials and Methods. A blot representative of three other experiments is shown. Right panel shows quantification of cytochrome *c* by densitometer. Data are the means $\pm$ SD value from three separate experiments.

Figure S3. Reduction of Bax or Bak protein level in Fig. 5.

Reduction of Bax or Bak protein level was confirmed by Western blotting with anti-Bax or anti-Bak antibodies. Quantification of Bax or Bak was performed by densitometer. Data are the means $\pm$ SD value from three separate experiments.

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Fig.1

Fig.2

Fig.3

Fig.4A

control

38 h

0 h

48 h

24 h

Fig.4B

Fig.5

Fig. 5

Fig.6

A

B

C

Fig. 7

A

B

Fig. 8

A

B

Fig.9

Fig. 10

Figure S1

Figure S2

A

B

Figure S3