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PSAP induces a unique Apaf-1 and Smac-dependent mitochondrial apoptotic pathway independent of Bcl-2 family proteins

Ting Li [a,b], Linlin Zeng [a,b], Wei Gao [a], Mei-Zhen Cui [a], Xueqi Fu [b], Xuemin Xu [a,*]

[a] Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996, USA
[b] Edmond H. Fischer Signal Transduction Laboratory, College of Life Sciences, Jilin University, Changchun 130012, China

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ABSTRACT

Presenilin-associated protein (PSAP) has been identified as a mitochondrial proapoptotic protein. However, the mechanism by which PSAP induces apoptosis remains unknown. To this end, we have established an inducible expression system. Using this system, we have examined the roles of B-cell lymphoma 2 (Bcl-2) family proteins, cytochrome c, Smac (Smac/Diablo, second mitochondria-derived activator of caspases/direct IAP binding protein with low PI), and Apaf-1 (apoptotic protease-activating factor) in PSAP-induced apoptosis. Our results demonstrate that knockdown of Apaf-1 abolished PSAP-induced caspase activation and poly(ADP ribose) polymerase (PARP) cleavage, indicating that the apoptosome formation triggered by cytochrome c is crucial for PSAP-induced apoptosis. Our data also demonstrate that knockdown of Smac abolished PSAP-induced caspase activation and PARP cleavage, indicating that, in addition to Apaf-1 or apoptosome formation, Smac is also essential for PSAP-induced apoptosis. However, interestingly, our data demonstrate that overexpression of Bcl-2 and Bcl-xL did not protect cells from PSAP-induced apoptosis, and that knockdown of Bid, Bax, and Bak had no effect on PSAP-induced cytochrome c and Smac release, indicating that PSAP-induced apoptosis is not regulated by Bcl-2 family proteins. These results strongly suggest that PSAP evokes mitochondrial apoptotic cascades via a novel mechanism that is not regulated by Bcl-2 family proteins, but that both the formation of cytochrome c-Apaf-1 apoptosome and the presence of Smac are absolutely required for PSAP-induced apoptosis.

1. Introduction

Apoptosis is a highly regulated and programmed form of cell death [1]. It not only plays critical roles in the regulation of normal development and homeostasis of multicellular organisms but is also critical in removing unwanted and potentially hazardous cells, such as tumor cells, virus-infected cells, and damaged cells; for review see [2]. Dysregulation of apoptosis has been implicated in the pathogenesis of several human diseases, such as cancer, autoimmunity, AIDS, and neurological disorders including Alzheimer's disease [3–6].

Apoptosis is primarily executed by a family of proteases known as caspases, which work by disassembling a cell [7,8]. Caspases are activated by proteolytic processing and are broadly divided into initiator caspases (e.g. procaspase-8 and -9) and executioner caspases (such as procaspase-3 and -7) [7]. Two major pathways have been identified to lead to caspase activation: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway [9]. In the extrinsic pathway, upon binding extracellular stimuli, the death receptor recruits adaptor protein FADD (Fas-associated death domain) and procaspase-8 to form the death-inducing signaling complex (DISC), leading to the activation of the initiator caspase, caspase-8. On the other hand, in the intrinsic pathway, dysfunction of mitochondria, caused by a variety of stress stimuli including UV radiation, DNA damage, growth factor deprivation, and viral virulence factors [10], results in the release of several proapoptotic proteins including cytochrome c and Smac (second mitochondria-derived activator of caspases, also known as DIABLO [direct inhibitor of apoptosis (IAP)-binding protein with low PI]). The released cytochrome c initiates the formation of the apoptosome through interaction with Apaf-1 (apoptotic protease-activating factor) and recruiting of procaspase-9 in the presence of dATP, leading to the activation of the other initial caspase, caspase-9 [11]. On the other hand, the Smac released from mitochondria activates caspase-9 via binding to IAP [12]. The activated initiator caspases, caspase-8 and

Abbreviations: PSAP, presenilin-associated protein; PARP, poly(ADP-ribose) polymerase; Bcl-2, B-cell lymphoma 2; LacZ, β-galactosidase; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; FADD, Fas-associated death domain; DISC, death-inducing signaling complex; Apaf-1, apoptotic protease-activating factor 1; Smac/DIABLO, second mitochondria-derived activator of caspases/direct IAP binding protein with low PI; IAP, inhibitors of apoptosis protein; COX-1, cytochrome c oxidase, siRNA, Small interfering RNA, HEK293, human embryonic kidney 293 cell line

* Corresponding author at: Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA. Tel.: +1 865 974 8206; fax: +1 865 974 5616.
E-mail address: xmx@utk.edu (X. Xu).

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caspase-9, in turn activate the executor caspase-3 and eventually lead to apoptosis. The release of these apoptotic factors, such as cytochrome c and Smac, is generally thought to be controlled by members of B-cell lymphoma 2 (Bcl-2) family proteins [13].

Presenilin-associated protein (PSAP) was originally identified as a protein interacting with presenilin, which is implicated in Alzheimer's disease pathogenesis. Subsequent study revealed PSAP as a novel mitochondrial apoptotic protein that induces apoptosis when it is overexpressed [14]. However, the details of the molecular mechanism by which PSAP induces apoptosis remain elusive. To elucidate these details, using caspase inhibitors and the small interfering RNA (siRNA) approach, we investigated the roles of the initiator caspase-8 and caspase-9, the mitochondrial apoptotic factors cytochrome c and Smac, the Bcl-2 family proteins, and Apaf-1 in PSAP-induced apoptosis. Our results clearly demonstrate that knockdown of either Apaf-1 or Smac had no effect on PSAP-induced cytochrome c release but completely blocked PSAP-induced apoptosis, suggesting that both apoptosome formation and elimination of IAP inhibition are crucial for PSAP-induced apoptosis. Inhibition of caspases blocked PSAP-induced, poly(ADP ribose) polymerase (PARP) cleavage, a sign of execution of apoptosis, but had no effect on PSAP-induced release of cytochrome c and Smac, indicating that the release of the proapoptotic factors from mitochondria is an earlier event in PSAP-induced apoptotic cascades. However, our result interestingly revealed that PSAP-induced apoptosis is not protected by overexpression of Bcl-2 and Bcl-xL and that the proapoptotic Bcl-2 proteins Bax, Bak, and Bid are not required for PSAP-induced apoptosis, suggesting PSAP induces a unique mitochondrial apoptotic pathway that is not controlled by the Bcl-2 family of proteins.

2. Materials and methods

2.1. Reagents

Lipofectamine 2000 transfection reagent, the ponasterone A-inducible gene expression system, and antibiotics G418 and zeocin were purchased from Invitrogen (Carlsbad, CA, USA). Specific siRNAs and HiPerfect transfection reagent were purchased from QIAGEN (Valencia, CA, USA). Ponasterone A was from A.G. Scientific (San Diego, CA, USA). Complete Protease Inhibitor Cocktail Tablets were purchased from Roche Applied Science (Indianapolis, IN, USA). Etoposide and the general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Streptolysin O and β-actin antibody were purchased from Sigma (St. Louis, MO, USA). Antibodies against PARP, caspase-3/7/8/9, Bcl-2, Bcl-xL, Bax, Bid, Bak, and Smac were purchased from Cell Signaling Technology (Danvers, MA, USA); antibodies against Apaf-1 and cytochrome c were purchased from BD Pharmingen. COX-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); both anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from GE Healthcare (Piscataway, NJ, USA).

2.2. Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Lonza, Walkersville, MA, USA), 100 units/mL penicillin (Lonza), and 100 μg/mL streptomycin (Lonza). For transfection of 293 cells with plasmids, the transfection reagent Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's instructions. For transfection of cells with siRNA, the HiperFect Transfection Reagent (QIAGEN) was used following the manufacturer's instructions. Stable cell lines were selected by addition of appropriate antibiotics as indicated in the text.

2.3. Establishment of ponasterone A-inducible expression system in HEK293 cells

The PSAP expression plasmid was constructed as described previously [14]. To establish an inducible expression system, PSAP cDNA was subcloned into the inducible pIND vector. HEK293 cells were first transfected with the regulator vector pVgRXR, which constitutively expresses both subunits of a functional ecdysone receptor from *Drosophila*, and a stable cell line was selected with Zeocin (400 μg/ml). The pVgRXR-stably transfected cells were then further transfected with the inducible vector pIND bearing the PSAP cDNA, and the double stably transfected cells were selected with both Zeocin (400 μg/mL) and G418 (400 μg/mL). Upon addition of the

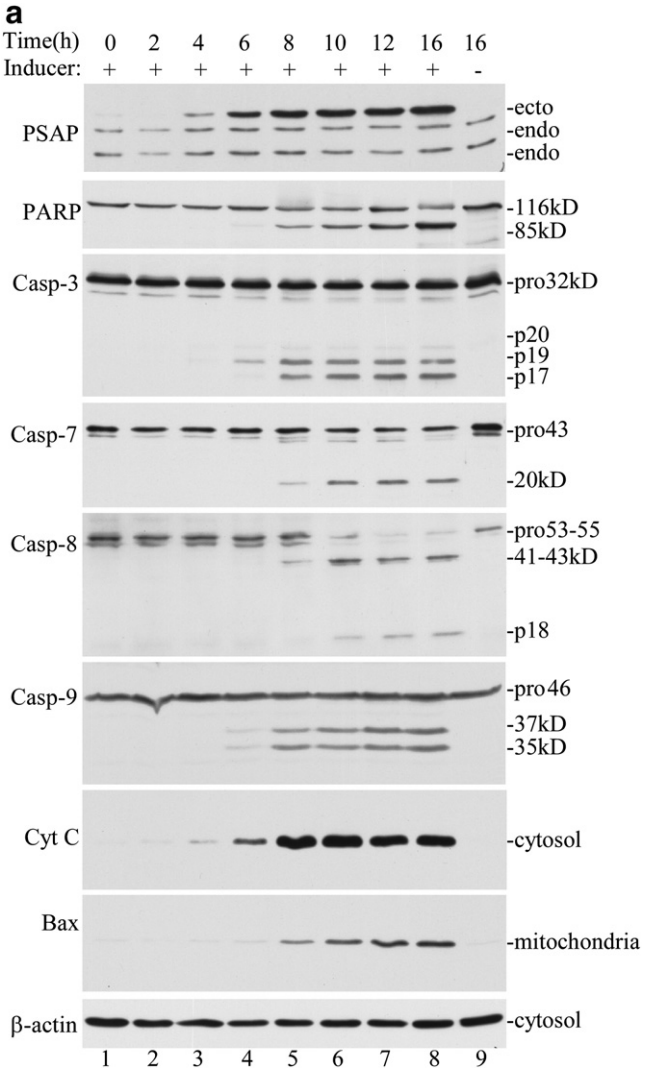

Fig. 1. Time course of PSAP-induced apoptosis. a. After induction of PSAP expression by addition of ponasterone A, cells were harvested at various time points, as indicated, and divided into two portions. Half the harvested cells was directly lysed and separated by SDS-PAGE, followed by Western blot analysis. The other half of the cells was used for preparation of cytosolic extracts and a mitochondria-containing membrane fraction as described in Materials and methods. *Top panel*, induction of PSAP expression. *Second panel*, PARP cleavage. *Third, fourth, fifth, and sixth panels*, activation of caspase-3, caspase-7, caspase-8, and caspase-9, respectively. Activation of caspases was determined by the formation of active forms of these caspases. One of these membranes was also re-probed with anti-actin antibody to indicate relative loading of samples (bottom panel). *Seventh panel*, cytochrome c release from mitochondria to cytosol. *Eighth panel*, Bax translocation from cytosol to mitochondria (mitochondria-containing membrane fraction). b. The profile of DNA content was determined by flow cytometric analysis. Left column, results from control cells; right column, results from the PSAP-expressing cells. Time is indicated in each panel.

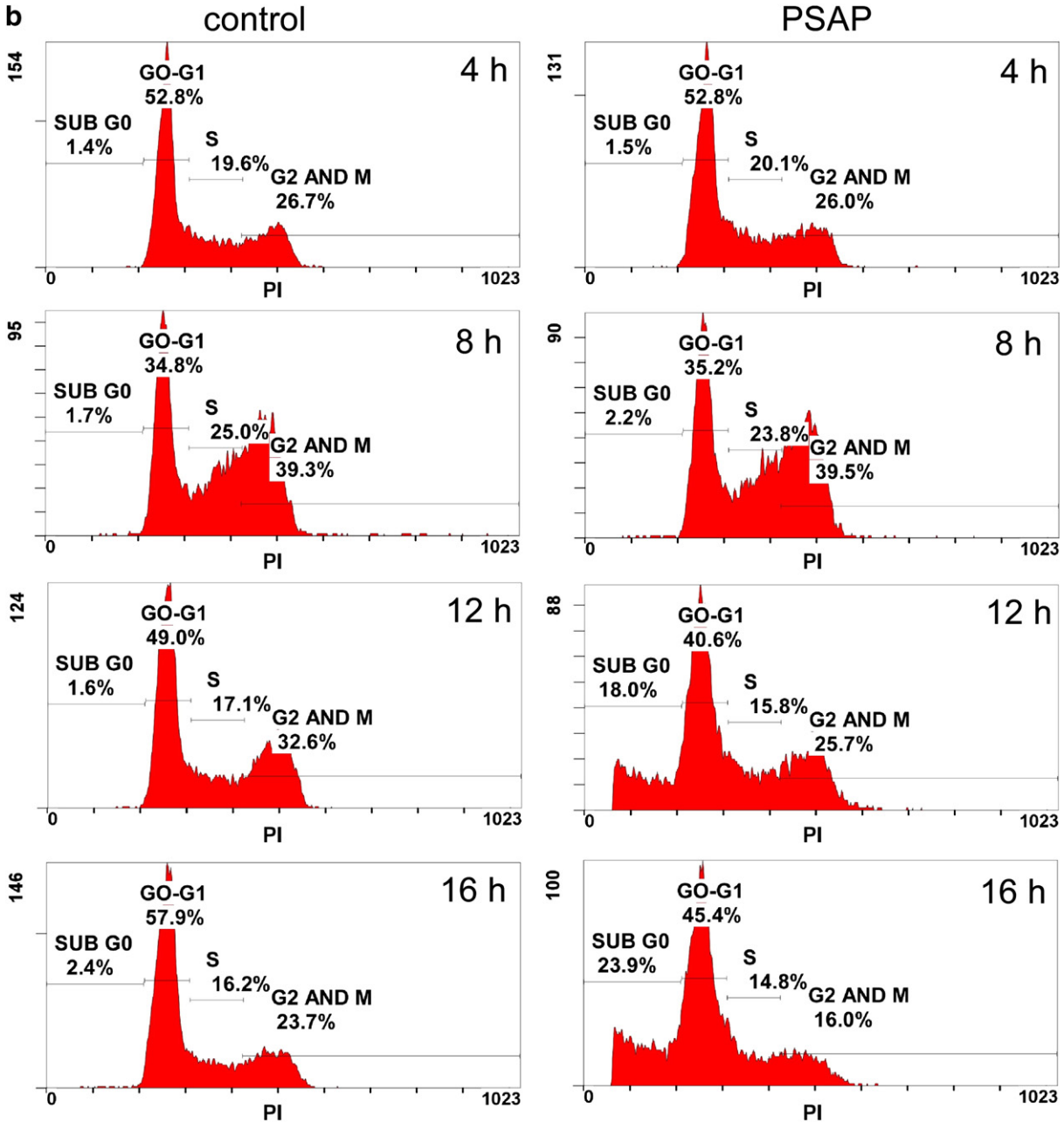

Fig. 1 (*continued*).

inducer ponasterone A, the functional ecdysone receptor binds upstream of the ecdysone responsive promoter in the pIND vector and activates expression of PSAP gene.

2.4. Establishment of a stable cell line with a functional Apaf-1-shRNA expression vector

The Apaf-1 shRNA expression vector from Sigma-Aldrich was transfected into HEK293 cells using Lipofectamine 2000, and the stable line was selected by addition of puromycin (5 μg/mL) following the manufacturer's instructions.

2.5. Subcellular fraction

For examination of cytochrome c and Smac release and Bax relocation, cytosolic extracts and mitochondria-containing fractions were prepared by permeabilizing cells with streptolysin O (Sigma-Aldrich) using the method described previously by Mosser et al. [15], with slight modification. Briefly, cells (10^6) were washed with PBS, collected by centrifugation, and resuspended in 10 μL of streptolysin O buffer (20 mM HEPES, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture) containing 60 units of streptolysin O. After incubation at 37 °C for 25 min, the permeabilization of cells was monitored by trypan blue staining. At the time when 95% cells were stained, permeabilized cells were pelleted by centrifugation at 16,000 ×*g* for 15 min at 4 °C. The supernatant containing the cytoplasmic fraction was then isolated from the pellet that contained the mitochondrial fraction. The purity of the cytoplasmic fraction was assessed by confirming the absence of cytochrome c oxidase 1 (COX-1) with Western analysis. The pellet was lysed in lysis buffer (50 mM Tris, pH 6.85, 8 M urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors) by sonication for 30 s. Both the cytosolic and mitochondrial fractions were

then subjected to SDS-polyacrylamide gel electrophoresis (10–15%) followed by Western blot analysis using antibodies against cytochrome c, Smac, COX-1, and Bax.

2.6. siRNA treatment

Small interfering RNA against Apaf-1, caspase-9, caspase-8, Smac, Bax, Bak, and Bid and the control non-silencing siRNA duplex that does not target any sequence in the genome were purchased from QIAGEN. Cells were transfected with these siRNAs three times at 2-day intervals using HiPerfect transfection reagent (QIAGEN), following the instructions provided by the manufacturer. On day 5, cells were either induced for PSAP expression by the addition of ponasterone A or transfected with a PSAP-expression plasmid as indicted in the text. Twenty-four hours after induction of PSAP expression or transfection with PSAP cDNA, cells were harvested. Half of the cells was lysed and directly subjected to SDS-PAGE followed by Western blot analysis; the other half of the cells was used to prepare cytosolic and mitochondrial fractions.

2.7. SDS-PAGE and Western blotting

For analysis of PARP cleavage, caspase activation, and the expression and subcellular localization of Apaf-1, PSAP, Bax, Bid, Bak, Smac and cytochrome c, cells and the mitochondria-containing membrane fraction were lysed by sonication for 20 s on ice in Western blot lysis buffer (50 mM Tris–HCl, pH 6.8, 8 M urea, 5% mercaptoethanol, 2% SDS, and protease inhibitor mixture). After addition of 4× SDS sample buffer and boiling at 100 °C for 7 min, samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 6% for Apaf-1, 9% for PARP and PSAP, 13% for Bcl-2 and Bcl-xL, 10–15% for caspases, COX-1, cytochrome c, Bax, Bid, Bak and Smac) and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica, USA). The membranes were probed with appropriate antibodies as described in figure legends. For the cytosolic fraction, samples were directly treated with 4× SDS sample buffer and subjected to SDS-PAGE followed by Western blot analysis as described previously [14].

2.8. Data analysis

All Western blots shown are representatives of five independent experiments. For statistical analysis, the density of each band was quantified using the Gel Digitizing Software UN-SCAN-IT (Silk Scientific, Orem, UT, USA). The ratios of protein levels between treated samples and controls are expressed as mean ± SEM, $n = 5$. A single comparison analysis was made using two-tailed unpaired Student t tests. For t tests, p values of ≤ 0.05 were considered statistically significant.

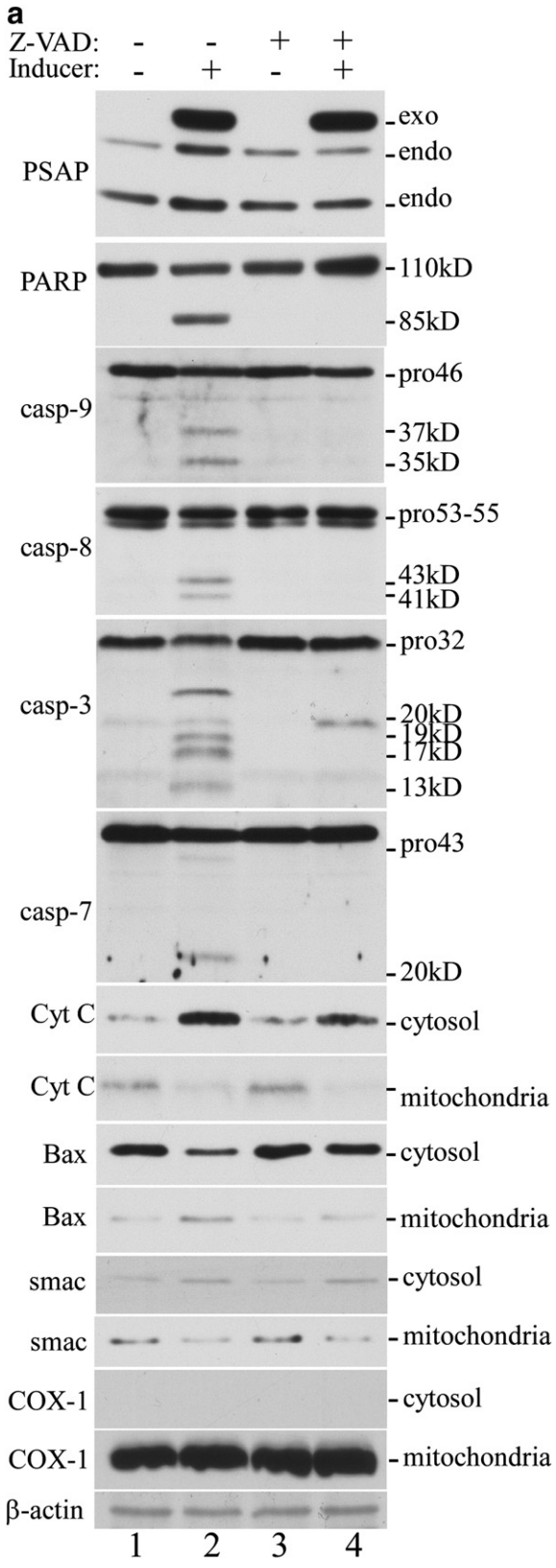

Fig. 2. General caspase inhibitor Z-VAD inhibited PSAP-induced apoptosis and caspase activation, but not cytochrome c release and Bax translocation. Cells were cultured in the presence or absence of 100 μM Z-VAD, the general caspase inhibitor. After induction of PSAP expression for 16 h, half the cells were directly lysed and subjected to Western blot analysis, and the other half were used for preparation of cytosolic extracts and mitochondria-containing membrane fraction. a. *Top panel*, ponasterone A induced PSAP expression (lanes 2 and 4). *Second panel*, upon induction of PSAP expression, PARP cleavage was detected in cells cultured in the absence of Z-VAD (lane 2), but not in cells cultured in the presence of Z-VAD (lane 4). *Third to sixth panels*, PSAP induced caspase activation in the absence of Z-VAD (lane 2), but not in the presence of Z-VAD (lane 4). *Seventh and eighth panels*, cytochrome c was released from mitochondria into the cytosol upon induction of PSAP expression regardless of whether cells were cultured in the presence or absence of Z-VAD (lanes 2 and 4). *Ninth and tenth panels*, PSAP-induced Bax translocation from cytosol to mitochondria was blocked by Z-VAD (compare lanes 4 with lane 2). *Eleventh and twelfth panels*, Smac was released from mitochondria to cytosol upon induction of PSAP expression regardless of whether cells were cultured in the presence or absence of Z-VAD (lanes 2 and 4). *Thirteenth and fourteenth panels*, reprobes of the eleventh and twelfth panels with anti-COX I antibody to confirm the mitochondria remained intact during preparation. The membrane in the second panel was also re-probed with anti-actin antibody to indicate relative loading of samples (bottom panel). b. Quantification and statistical analysis of the release of cytochrome c and Smac and translocation of Bax under different conditions. Single, double, and three asterisks indicate significant differences between control and treatment at $p<0.05$ 0.01, and 0.001 respectively.

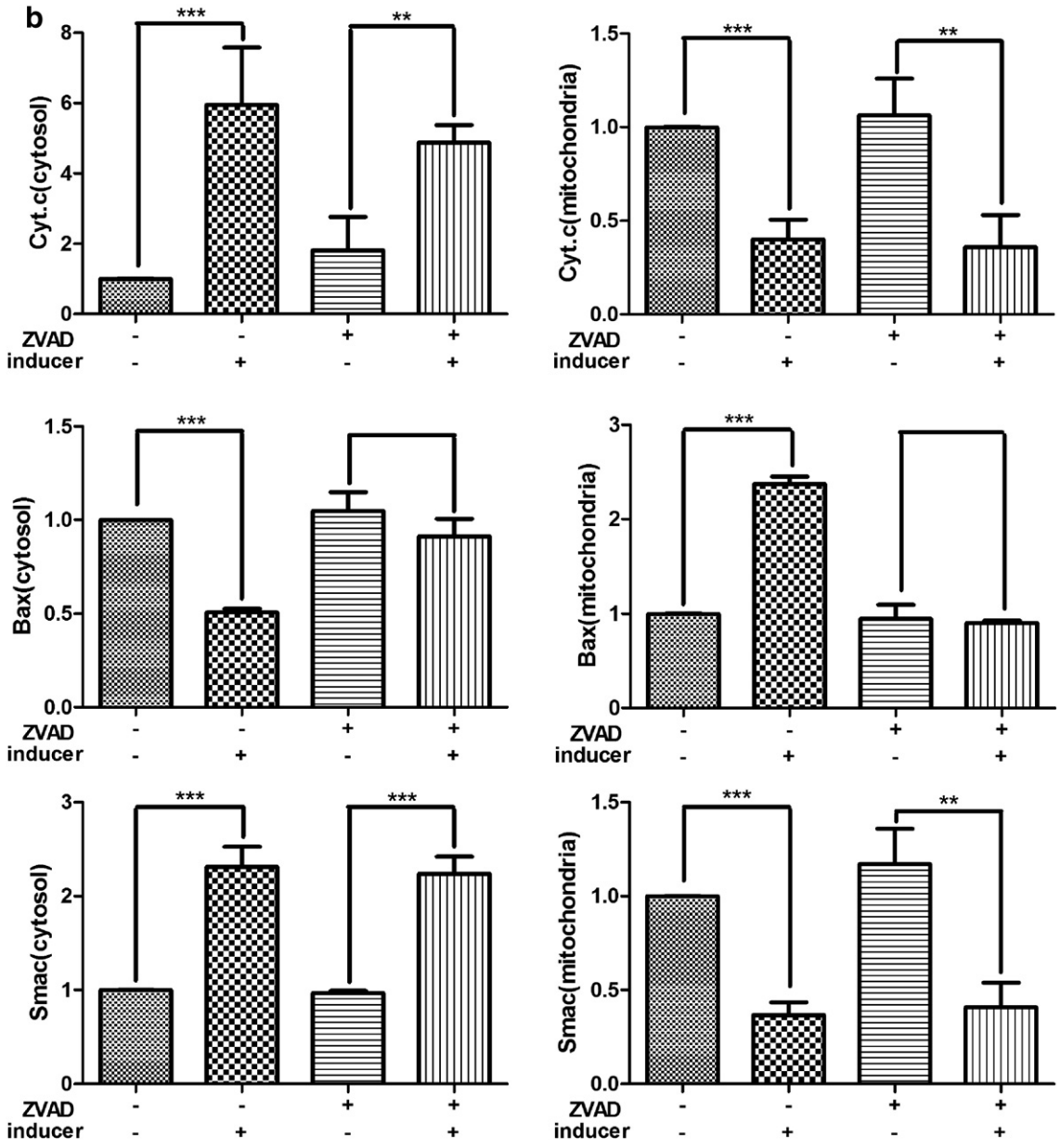

Fig. 2 (*continued*).

2.9. Flow cytometry

A flow cytometric assay was performed as described previously [14].

3. Results

3.1. Time course of PSAP-induced apoptosis

To determine the mechanism by which PSAP induces apoptosis, we have established an Ecdysone-Inducible Expression System (with ponasterone A) [16] for inducible expression of PSAP in HEK293 cells; we performed a time-course experiment to analyze apoptosis upon induction of PSAP. At various time points, equal numbers of cells were harvested, and the expression of PSAP, cleavage of PARP, activation of caspases, and the translocation of mitochondria pro-apoptotic proteins were analyzed by Western blot. As reported in our previous study, PSAP is expressed in two isoforms as a result of alternative splicing, and both isoforms are equally apopototic active [17]. As shown in the top panel of Fig. 1a, 4 h after addition of the inducer ponasterone A, the induced expression of PSAP became detectable. Note that the recombinant PSAP had a slightly slow migration rate; this is because the recombinant PSAP was expressed with a myc-tag fused to its C-terminal [17]. At 8 h PSAP induction, the 85-kDa fragment of PARP, which is characteristic of apoptosis, became detectable and increased in a time-dependent manner (second panel). Activation of caspase-9 (sixth panel) and caspase-3 (third panel) and cytochrome c release (seventh panel) were clearly detected at an earlier time point, 4 h after induction, but the activation of caspase-8 (fifth panel) and caspase-7 (fourth panel) was detected at later time points, 10 and 8 h after induction, respectively. Likewise, Bax translocation to the mitochondria (eighth panel) was detected at a later time point 8 h after induction. Since PSAP expression, cytochrome c release, caspase activation, and PARP cleavage all reached their peak value at 16 h after induction, the following experiments that used this inducible system were carried out at 16-h time points.

The time course of PSAP-induced apoptosis was also analyzed by determining DNA fragmentation. At various time points, cells were stained with propidium iodide followed by flow cytometric assay. As shown in Fig. 1b, upon induction of PSAP for 12 h, a significant portion of cells displayed reduced DNA content as demonstrated by the appearance of the hypodiploid DNA, the sub-Go peak (18%) at the left of the Go/G1 peak. Cells with lighter DNA staining than that of G1 cells are considered apoptotic [18]. The percentage of cells associated with the sub-Go peak was 24% at the 16-h point in PSAP-overexpression cells and less than 2% in the control cells.

3.2. General caspase inhibitor Z-VAD inhibited PSAP-induced apoptosis and Bax translocation, but not cytochrome c and Smac release

As shown in Fig. 1, PSAP-induced apoptosis involves caspase activation and cytochrome c release. To determine the roles of the caspases in PSAP-induced apoptosis and the translocation of mitochondrial proapoptotic factors, the effect of the general caspase inhibitor Z-VAD, which irreversibly inhibits all caspase activation, on PSAP-induced apoptosis was examined. As shown in Fig. 2a, upon induction of PSAP expression at 16 h, PARP cleavage and caspase activation were clearly observed (lane 2, panels 1 to 6). As expected, upon induction of PSAP, the level of cytochrome c in the cytosol dramatically increased with a concomitant decrease in the mitochondrial fraction (lane 2, panels 7 and 8). Similarly, induction of PSAP resulted in a decrease of Bax in the cytosol, and an increase in the mitochondrial fraction was clearly detected (lane 2, panels 9 and 10). In addition, upon induction of PSAP, mitochondrial release of the other important mitochondrial proapoptotic factor Smac was also detected (lane 2, panels 11 and 12). When the cells were treated with the general caspase inhibitor Z-VAD (100 μM) for 30 min prior to induction of PSAP, the PSAP-induced apoptosis was blocked as determined by the blockage of caspase activation (lane 4, panels 3 to 6) and the disappearance of PARP cleavage (lane 4, second panel). It was also noted that in the presence of the caspase inhibitor, Bax translocation from cytosol to mitochondria was also blocked (lane 4, panels 9 and 10), indicating that Bax translocation depended on the activation of caspase. However, as shown in lane 4 of panels 7, 8, 11, and 12, the release of cytochrome c and Smac from mitochondria was not affected by inhibition of caspase activity. This result strongly suggests that the releases of cytochrome c and Smac from the mitochondria were independent of caspase activation and are the early events in

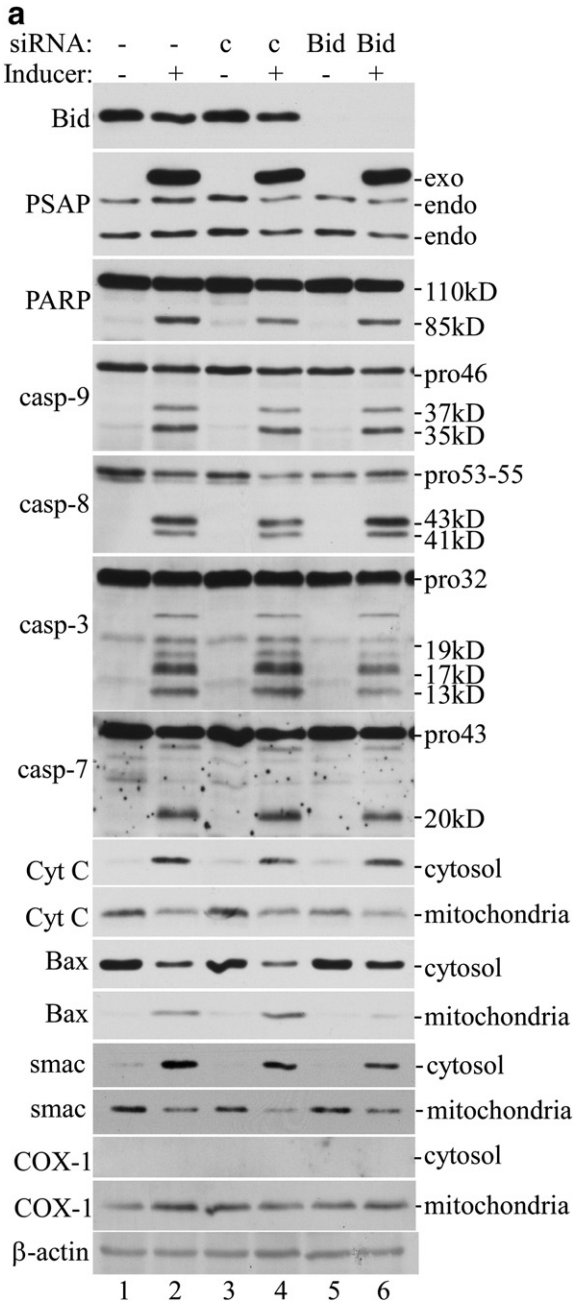

Fig. 3. Bcl-2 family proteins had no regulatory effects on PSAP-induced apoptosis. Lanes 5 and 6 show the successful knockdown of Bid (A), Bax (C), Bak (E) individually, and both Bax and Bak together (G). Since the molecular weight of Bak (25 kDa) is slightly higher than that of Bax (20 kDa), in the double knockdown cells, Bax and Bak were probed with a mixture of both anti-Bax and anti-Bak antibodies. a. Knockdown of Bid had no effect on PSAP-induced caspase activation, PARP cleavage, or cytochrome c release but blocked the translocation of Bax (compare lane 6 with lanes 2 and 4, third to eleventh panels). b. Quantification and statistical analysis of the Western blot results for the release of cytochrome c and Smac and the translocation of Bax shown in A. Single, double, and three asterisks indicate significant differences between control and treatment at $p<0.05$ 0.01, and 0.001 respectively. c. Knockdown of Bax had no effect on PSAP-induced caspase activation, PARP cleavage, or the translocation of cytochrome c and Smac (compare lane 6 with lanes 2 and 4, third to eleventh panels). *Twelfth and thirteenth panels*, reprobes of the membranes in the tenth and eleventh panels with anti-COX I antibody to confirm the mitochondria remained intact during preparation. The membrane in the second panel was also re-probed with anti-actin antibody to indicate relative loading of samples (bottom panel). d. Quantification and statistical analysis of the Western blot results for the release of cytochrome c and Smac shown in c. Single, double, and three asterisks indicate significant differences between control and treatment at $p<0.05$ 0.01, and 0.001 respectively. e and g. Knockdown of Bak and double knockdown of Bax and Bak had no effect on PSAP-induced PARP cleavage, caspase activation, or the release of cytochrome c and Smac (compare lane 6 with lanes 2 and 4, third to eleventh panels). In this set (e and g) of experiments, cells in lanes 2, 4, and 6 were transfected with PSAP cDNA (second panel); cells in lanes 1, 3, and 5 were transfected with a plasmid expressing LacZ (bottom panel). F and H are the quantification and statistical analysis of the Western blot results for the release of cytochrome c and Smac in Bak-knockdown (e) and Bak/Bax-double knockdown (g) cells, respectively. Single, double, and three asterisks indicate significant differences between control and treatment at $p<0.05$ 0.01, and 0.001 respectively. i. Overexpression of Bcl-2 and Bcl-xL proteins had no effect on PSAP-induced apoptosis. PSAP-inducible expression cells in lanes 1 and 2 were transfected with empty vector; cells in lanes 3 and 4 were transfected with a plasmid-expressing Bcl-2 (top panel); cells in lanes 5 and 6 were transfected with a Bcl-xL-expressing plasmid (second panel). The third panel was probed for inducible expression of PSAP; the fourth panel was probed for cleavage of PARP; fifth to eighth panels were probed for caspase activation; ninth to thirteenth panels were probed for cytochrome c, Smac, and COX-1 in the cytosolic and mitochondrial fractions; twelfth panel was probed for β-actin. j. Quantification and statistical analysis of the Western blot results for the release of cytochrome c and Smac shown in i. k. Single, double, and three asterisks indicate significant differences between control and treatment at $p<0.05$ 0.01, and 0.001 respectively. Overexpression of Bcl-2 blocked etoposide-induced apoptosis. l. Knockdown of Bax and Bak blocked etoposide-induced apoptosis.

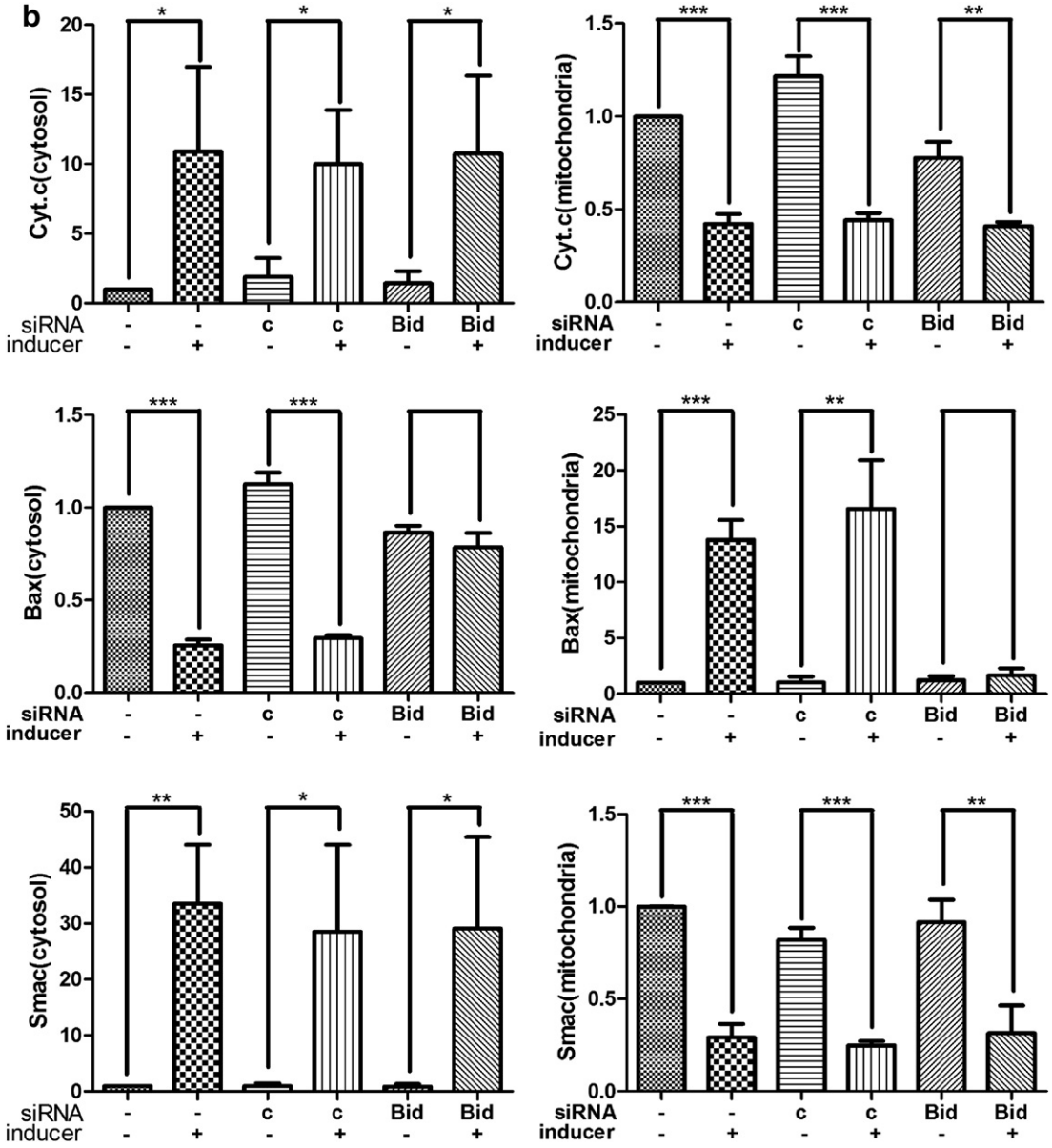

Fig. 3 (*continued*).

PSAP-induced apoptotic response. For mitochondrial apoptotic factors cytochrome c, Smac/Diablo, and Bax, the Western blot results of five independent experiments were quantified and normalized to the level of β-actin. As shown in Fig. 2b, regardless of presence or absence of Z-VAD, PSAP-induced cytochrome c (top row) and Smac (bottom row) releases were significant. However, in contrast with absence of Z-VAD, no significant translocation of Bax from cytosol to mitochondria was observed in the presence of Z-VAD (middle row).

3.3. The proapoptotic Bcl-2 family proteins are not required for PSAP-induced apoptosis

To determine whether the proapoptotic Bcl-2 proteins play a role in PSAP-induced apoptosis, the effects of knockdown of these proteins on PSAP-induced apoptotic events were explored. To knockdown these proteins, cells were transfected with siRNAs specific to Bax, Bak, and Bid using HiPerfect transfection reagent (QIAGEN), following the instructions provided by the manufacture as described in "Materials and methods." To determine the effect of knockdown of Bax and Bid, cells that inducibly express PSAP were used. Since knockdown of Bak attenuates inducible expression of PSAP for an unknown reason, the effects of knockdown of Bak and Bax/Bak double knockdown were examined in native HEK293 cells. As shown in Fig. 3a, after 5 days' treatment with siRNA against Bid, Bid expression was completely knocked down (lanes 5 and 6, top panel). In contrast, when the cells were treated with non-silencing control siRNA, the expression level of Bid and the PSAP-induced apoptotic response were not affected in comparison with cells treated with only transfection reagent (compare lane 4 with lane 2 through all the panels). As shown in the third panel, knockdown of Bid had almost no effect on PSAP-induced PARP cleavage (compare lane 6 with lanes 2 and 4). As discussed below, the slight decrease in the level of cleavage of PARP may reflect the effect of the attenuation of the amplification loop mediated by caspase-8 and tBid. As shown in panels 4 to 7, knockdown of Bid had no significant effect on PSAP-induced caspase activation (compare lane 6 with lanes 2 and 4). As shown in panels

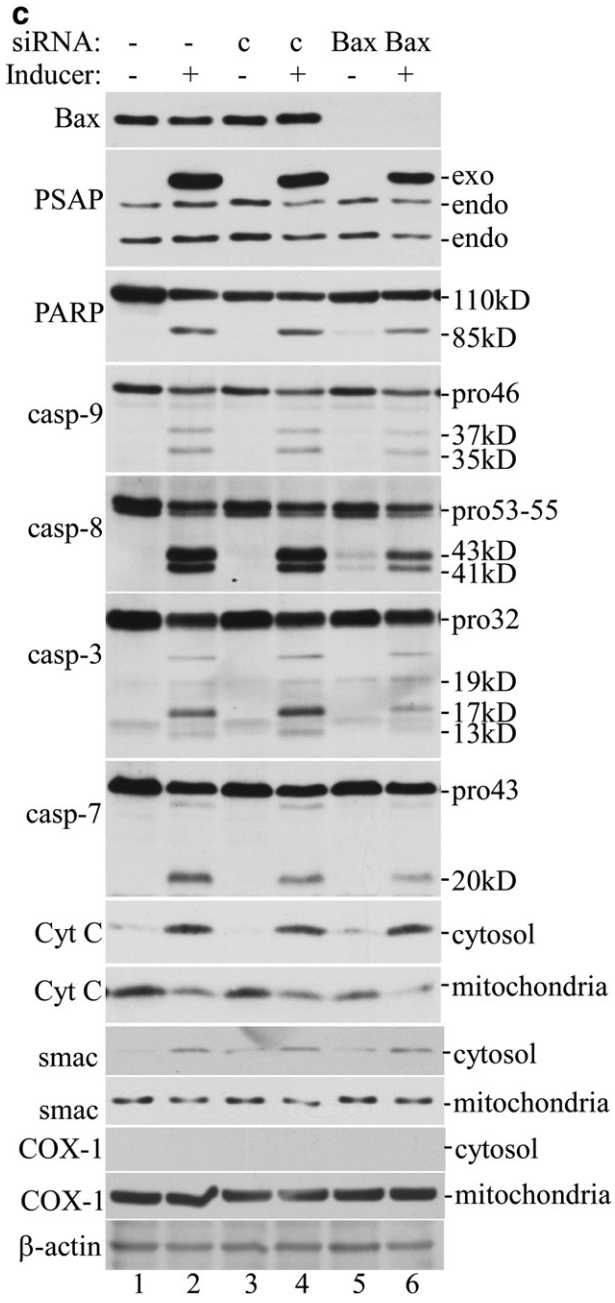

Fig. 3 (*continued*).

8, 9, 12, and 13, knockdown of Bid had no significant effect on PSAP-induced release of cytochrome c and Smac/Diabio from mitochondria into the cytosol (compare lane 6 with lanes 2 and 4). However, as shown in panels 10 and 11, knockdown of Bid almost completely blocked Bax translocation from cytosol to mitochondria (compare lane 6 with lanes 2 and 4). Results of the Western blot analysis for cytochrome c, Smac/Diablo, and Bax were quantified and normalized to the level of β-actin. As shown in Fig. 3b, regardless of Bid knockdown, PSAP-induced cytochrome c (top row) and Smac (bottom row) releases were significant. However, in contrast with non-silencing siRNA treated cells, no significant translocation of Bax from cytosol to mitochondria was observed in cells treated with Bid-specific siRNA (middle row). These results clearly indicate that PSAP-induced apoptotic events, including caspase activation, PARP cleavage, and cytochrome c release, were Bid independent. However, Bid was required for PSAP-induced Bax translocation.

The results presented above that knockdown of Bid blocked Bax translocation but had no effect on apoptosis suggest that Bax is not required for PSAP-induced apoptosis. To address this issue, we determined the effect of knockdown of Bax on PSAP-induced apoptosis. As shown in the top panel of Fig. 3c, after 5 days' treatment with Bax-specific siRNA, Bax was completely knocked down (lanes 5 and 6). As shown in the third panel, knockdown of Bax had almost no effect on PSAP-induced PARP cleavage (compare lane 6 with lanes 2 and 4). As mentioned above, the slight decrease in the level of cleavage of PARP may reflect the effect of the attenuation of the amplification loop mediated by caspase-8, tBid, and Bax. As shown in panels 4 to 7, knockdown of Bax had no effect on PSAP-induced caspase activation, nor did it have a significant effect on PSAP-induced releases of cytochrome c and Smac (panels 8 to 11, compare lane 6 with lanes 2 and 4). Western blot results for cytochrome c and Smac/Diablo were quantified and normalized to the level of β-actin. As shown in Fig. 3d, regardless of Bax knockdown, PSAP-induced cytochrome c (top row) and Smac (bottom row) releases were significant. These results clearly indicate that PSAP-induced apoptotic events, including caspase activation, PARP cleavage, and mitochondrial proapoptotic factor release, were Bax independent.

Next, we determined the effect of knockdown of Bak, a close relative of Bax, on PSAP-induced apoptosis. Since treatment with siRNA specific to Bak resulted in the impairment of inducible expression of PSAP for an unknown reason, knockdown of Bak was performed in wild type HEK293 cells. As shown in Fig. 3e, Bak expression was completely knocked down after 5 days of treatment with Bak-specific siRNA (lanes 5 and 6, top panel). At the time point when knockdown of Bak was confirmed, the cells were transfected either with PSAP expression plasmid or with β-galactosidase (LacZ) expression plasmid for 24 h, and the exogenous expression of PSAP (lanes 2, 4, and 6, second panel) and LacZ (lanes 1, 3, and 5, bottom panel) were detected by Western blot using specific antibodies. As shown in the third panel, upon transfection with PSAP cDNA, PARP cleavage was clearly detected in cells treated with transfection reagent only (lane 2), in cells treated with a non-silencing control siRNA (lane 4), and in cells treated with Bak-specific siRNA (lane 6). As shown in panels 4 to 7, PSAP-induced caspase activation was detected in all PSAP-transfected cells regardless of whether Bak was knocked down (compare lane 6 with lanes 2 and 4). As shown in panels 8 to 11, knockdown of Bak had no effect on PSAP-induced release of cytochrome c and Smac (compare lane 6 with lanes 2 and 4). Quantitative analysis of Western blot results for cytochrome c and Smac/Diablo revealed that regardless of Bak knockdown, PSAP-induced cytochrome c (top row) and Smac (bottom row) releases were significant (Fig. 3f). These results indicate that similar to Bax, Bak was not required for PSAP-induced apoptosis.

The data presented above demonstrate that neither Bax nor Bak was required for PSAP-induced apoptosis when they were knocked down individually. We next determined the effect of double knockdown of Bax and Bak on PSAP-induced apoptosis. As shown in Fig. 3g, both Bax and Bak were completely knocked down after 5 days' treatment with Bax- and Bak-specific siRNAs (lanes 5 and 6, top panel). As shown in the third to seventh panels, upon transfection with PSAP cDNA, PARP cleavage and caspase activation were detected in all cell lines regardless of whether Bax/Bak were knocked down (compare lane 6 with lanes 2 and 4, panels 3 to 7). As shown in panels 8 to 11, knockdown of Bax and Bak had no effect on PSAP-induced release of cytochrome c and Smac (compare lane 6 with lanes 2 and 4). Quantitative analysis of Western blot results for cytochrome c and Smac/Diablo revealed that regardless of Bak and Bak knockdown, PSAP-induced cytochrome c (top row) and Smac (bottom row) releases are significant (Fig. 3h). These data clearly indicate that both Bax and Bak were dispensable for PSAP-induced apoptosis.

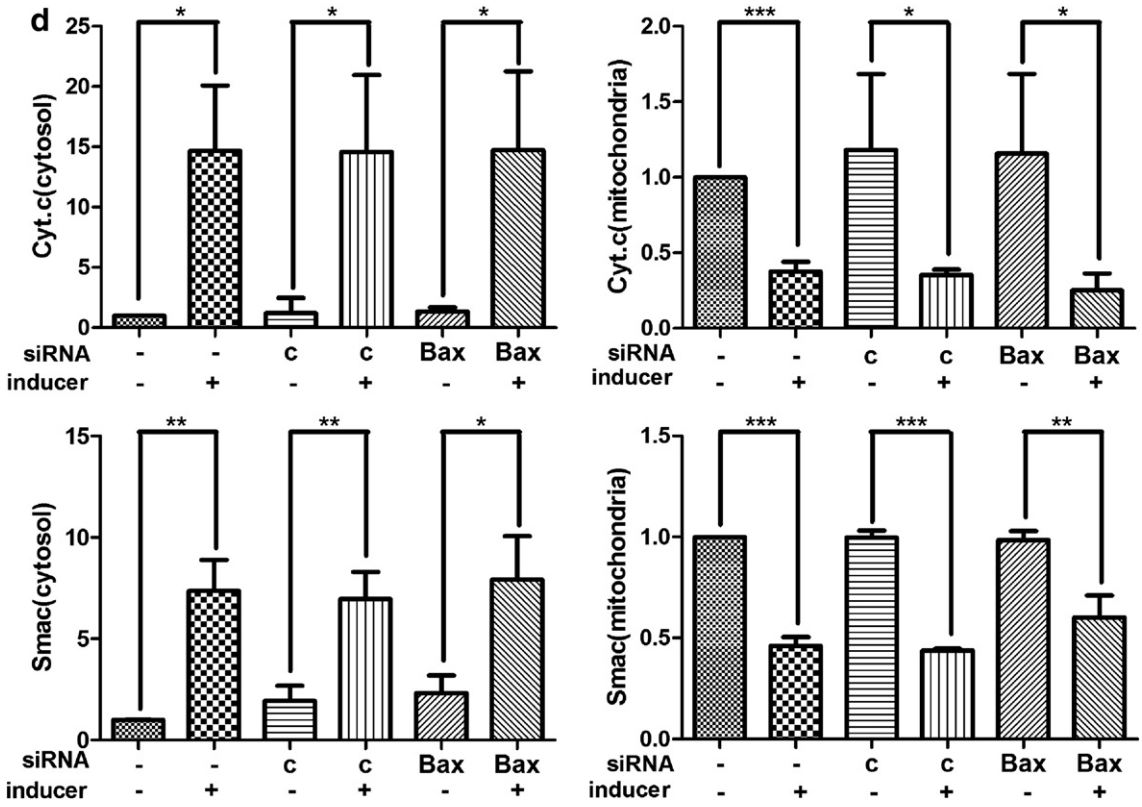

Fig. 3 (*continued*).

3.4. Anti-apoptotic Bcl-2 and Bcl-xL have no regulatory effects on PSAP-induced apoptosis

The above results clearly indicate that the proapoptotic Bcl-2 proteins are not required for PSAP-induced apoptosis. Next, we determined whether PSAP-induced apoptosis is regulated by anti-apoptotic Bcl-2 protein. As shown in Fig. 3i and j, overexpression of Bcl-2 and Bcl-xL had no effect on PSAP-induced apoptosis, as determined by PARP cleavage and the release of cytochrome c and Smac/Diablo. Taken together, these observations strongly suggest that PSAP-induced apoptosis was not regulated by Bcl-2 family proteins.

As control, we have performed experiments to determine the effects of Bcl-2 family protein on apoptosis induced by etoposide, which is a known intrinsic apoptotic inducer and induces Bax- and Bak-dependent apoptosis regulated by Bcl-2 family proteins [19]. HEK 293 cells transfected with either Bcl-2 cDNA or with siRNAs specific to Bax and Bak were treated with 45 μM etoposide (VP-16) for 24 h to induce apoptosis. As shown in Fig. 3 k and l, etoposide-induced apoptosis was completely inhibited by either overexpression of Bcl-2 protein or knockdown of Bax and Bak as reported by previous study [19]. This result confirms our experimental conditions as accurate.

3.5. Knockdown of caspase-9, but not caspase-8, abolished PSAP-induced apoptosis

The results presented above indicate that inhibition of caspase activation with the general caspase inhibitor Z-VAD had no effect on PSAP-induced release of either cytochrome c or Smac, but blocked PSAP-induced PARP cleavage. This result clearly indicates that the execution of PSAP-induced apoptosis depended on caspase activation. However, this set of data did not answer the question of which caspase is the initiator caspase in PSAP-induced apoptotic cascades. Next, we determined the role of caspase-9, the initiator caspase of the mitochondrial pathway, and caspase-8, the initiator caspase in the death receptor pathway, in PSAP-induced apoptosis. To do so, HEK293 cells expressing inducible PSAP were treated with caspase-9- or caspase-8-specific siRNA. As shown in Fig. 4, both caspase-9 and caspase-8 expression was completely suppressed in cells treated with specific siRNAs (lanes 5 and 6, top panel, Fig. 4a and b), but not in cells treated with either reagent only or non-silencing siRNA (lanes 1 to 4, Fig. 4a and b). As shown in Fig. 4a, upon induction of PSAP expression, PARP cleavage was detected in control cells (third panel, lanes 2 and 4) but not in cells treated with caspase-9 siRNA (lane 6). As shown in panels 4 and 5, knockdown of caspase-9 abolished the activation of caspase-3 and caspase-8 (compare lane 6 with lanes 2 and 4). In contrast, as shown in Fig. 4b, knockdown of caspase-8 had only a slight effect on PSAP-induced PARP cleavage and the activation of other caspases (compare lane 6 with lanes 2 and 4, panels 3 to 6). These results strongly indicate that caspase-9 was the initiator caspase in PSAP-induced apoptotic cascade. The slight decrease in the level of cleavage of PARP and the activation of other caspases in caspase-8 knockdown cells may reflect the effect of the abolishment of the amplification loop mediated by caspase-8 and tBid as discussed above.

3.6. Knockdown of Apaf-1 abolished PSAP-induced apoptosis, caspase activation, and Bax translocation, but not cytochrome c release

The results presented above clearly indicate that caspase-9 is crucial in the activation of other caspases and the subsequent cleavage of PARP. Next, we determined the role of apoptotic protease-activating factor 1 (Apaf-1), which is a key molecule for the activation of caspase-9 in the apoptosome, in PSAP-induced apoptosis. HEK293 cells were stably transfected with an Apaf-1-targeting shRNA expression plasmid as described in Materials and methods. As shown in

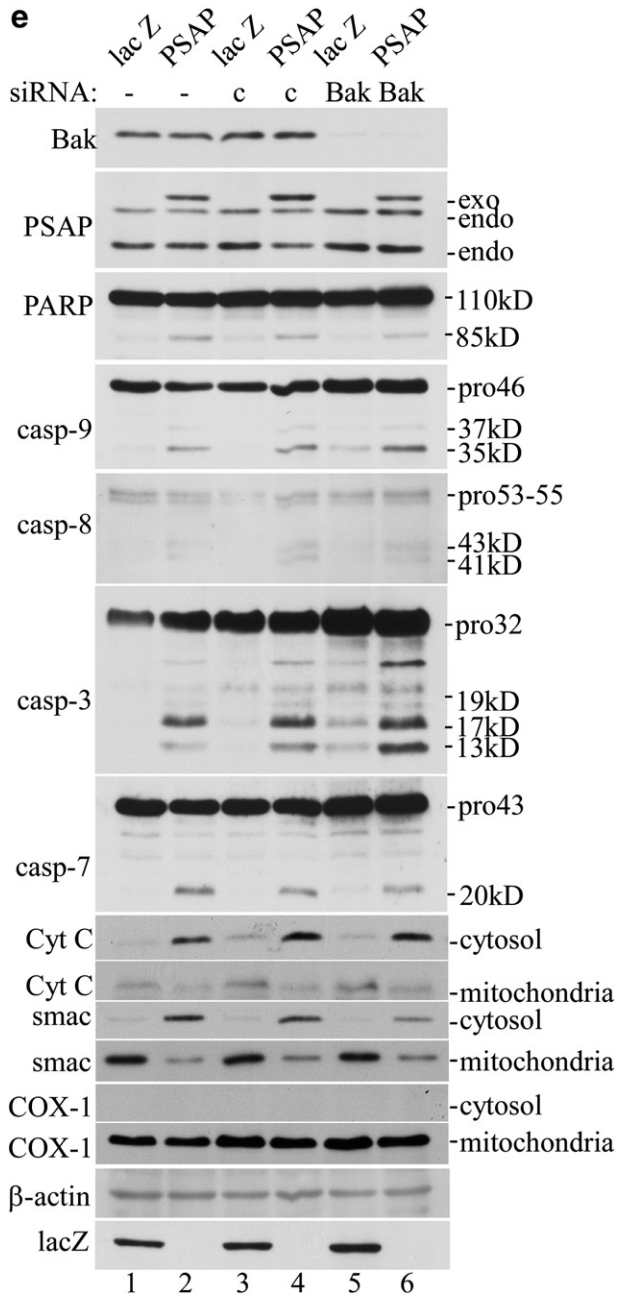

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Fig. 5a, Apaf-1 expression was completely suppressed in cells stably transfected with Apaf-1-targeting shRNA (lanes 3 and 4) but not in scrambled shRNA-transfected cells (lanes 1 and 2). When these cells were further transfected with LacZ or PSAP expression plasmid, as shown in the third panel, PARP cleavage was detected in scrambled shRNA-expressing cells (lane 2) but not in cells expressing shRNA against Apaf-1 (lane 4). As shown in panels 4 to 7, knockdown of Apaf-1 expression attenuated caspase activation induced by PSAP expression (compare lane 4 with lane 2). These results indicate that Apaf-1 was required for PSAP-induced apoptosis. As expected, as shown in panels 8 and 9, knockdown of Apaf-1 had no effect on PSAP-induced cytochrome c release from mitochondria (compare lane 4 with lane 2). In contrast, as shown in panels 10 and 11, knockdown of Apaf-1 completely blocked PSAP-induced Bax translocation (compare lane 4 with lane 2). Quantitative analysis of Western blot results for cytochrome c release and Bax translocation are shown in Fig. 5b.

3.7. Smac is indispensable for PSAP-induced apoptosis

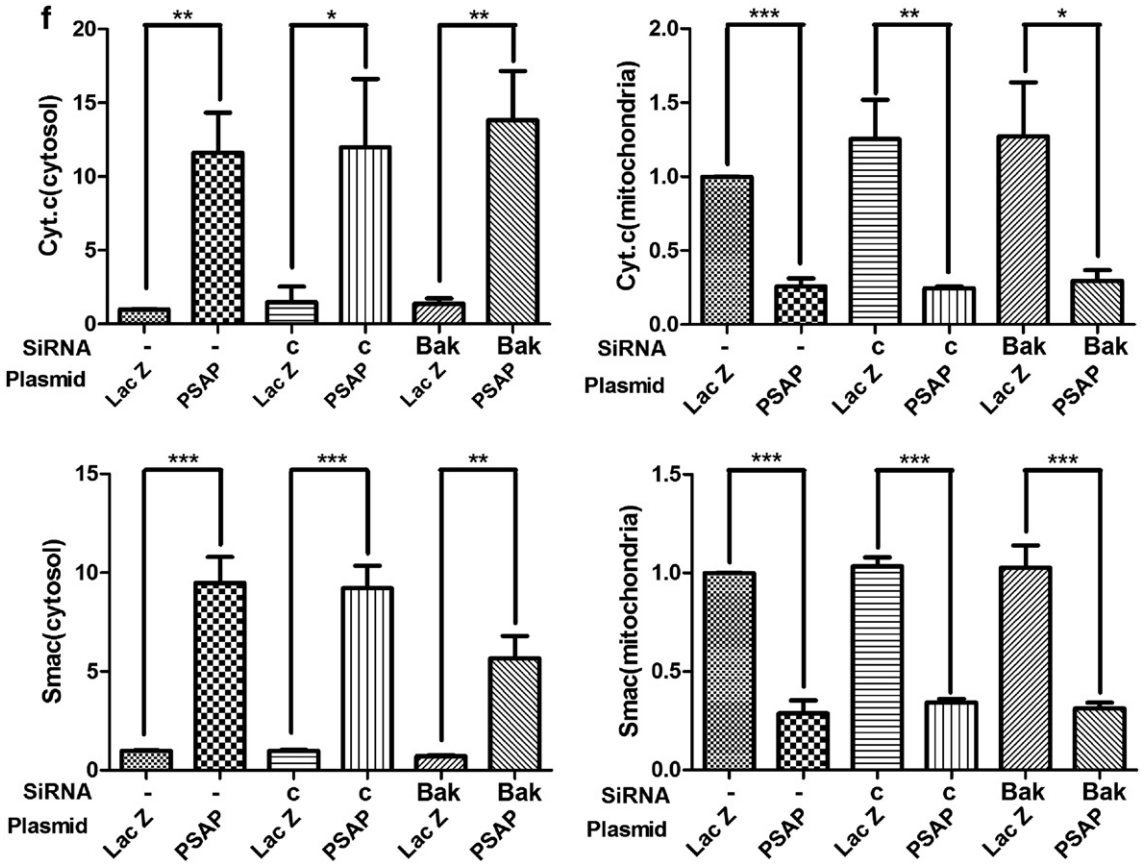
Finally, we examined the role of Smac, which is the other key mitochondrial proapoptotic factor promoting caspase-9 activation, in PSAP-induced apoptosis using an siRNA approach. As shown in Fig. 6a, treatment of cells that express inducible PSAP with Smac-specific siRNA completely suppressed the expression of Smac (lanes 5 and 6). As shown in panels 3 to 7, knockdown of Smac completely abolished PSAP-induced apoptotic events including PARP cleavage and caspase activation (compare lane 6 with lanes 2 and 4). However, similar to Apaf-1, knockdown of Smac had no significant effect on PSAP-induced cytochrome c release (panels 8 and 9), but blocked Bax translocation (panels 10 and 11). Quantitative analysis of Western blot results for cytochrome c release and Bax translocation are shown in Fig. 6B. These results indicate that both Apaf-1 and Smac were indispensable for PSAP-induced apoptosis.

4. Discussion

In a previous study, we reported that PSAP is a mitochondrial proapoptotic protein that causes apoptosis when it is overexpressed [14]. In the present study, using various approaches, including employment of inhibitors and siRNA technology, we investigated the mechanism by which PSAP induces apoptosis. Our results clearly indicate that PSAP induces apoptosis though a unique mitochondrial pathway that is not controlled by the Bcl-2 family proteins. In contrast, both Apaf-1 and Smac are required for PSAP-induced apoptosis.

The apoptotic process is executed by a group of cysteine proteases known as caspases that specifically cleave their substrates at aspartic acid residues [20]. As shown in Fig. 7, caspases are activated through two major pathways, i.e., the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway. In the death receptor pathway, activation of the initiator caspase, caspase-8, is mediated by the formation of DISC triggered by ligand binding to death receptors, while in the mitochondrial pathway the initiator caspase, caspase-9, is activated by the formation of apoptosome initiated by the release of cytochrome c and its subsequent binding to Apaf-1. The activated initiator caspases in turn activate the executor/effector caspases, such as caspase-3, leading to apoptosis via degradation of the nucleus and other intracellular structures [21]. In some types of cells known as Type I cells, death receptor-induced activation of the initiator caspase, caspase-8, can directly lead to the activation of the effector caspases. On the other hand, in the great majority of cells (Type II cells), the level of death receptor-induced activation of caspase-8 is less efficient for direct activation of downstream caspases, but is enough to promote the cleavage of Bid, a member of the Bcl-2 family protein. The cleaved Bid (tBid, truncated Bid) then induces cytochrome c release from mitochondria in synergy with Bax and Bak, the other two proapoptotic members of the Bcl-2 family protein [21]. Thus, mitochondria play a critical role in both intrinsic and extrinsic apoptotic pathways. Hence, as a proapoptotic protein, the mitochondrial localization of PSAP places it in an important position, and this prompted us to determine the mechanism by which PSAP induces apoptosis.

By using an inducible expression system, our data demonstrate that overexpression of PSAP induces a series of apoptotic events including PARP cleavage, caspase activation, the release of cytochrome c and Smac from mitochondria, and the translocation of Bax to the mitochondria. The data from the time course experiment also demonstrate that cytochrome c release occurs prior to caspase activation, suggesting that cytochrome c release is an earlier event in PSAP-induced apoptotic cascades. This view is further supported by the finding that inhibition of caspase activation completely blocked PSAP-induced PARP cleavage,



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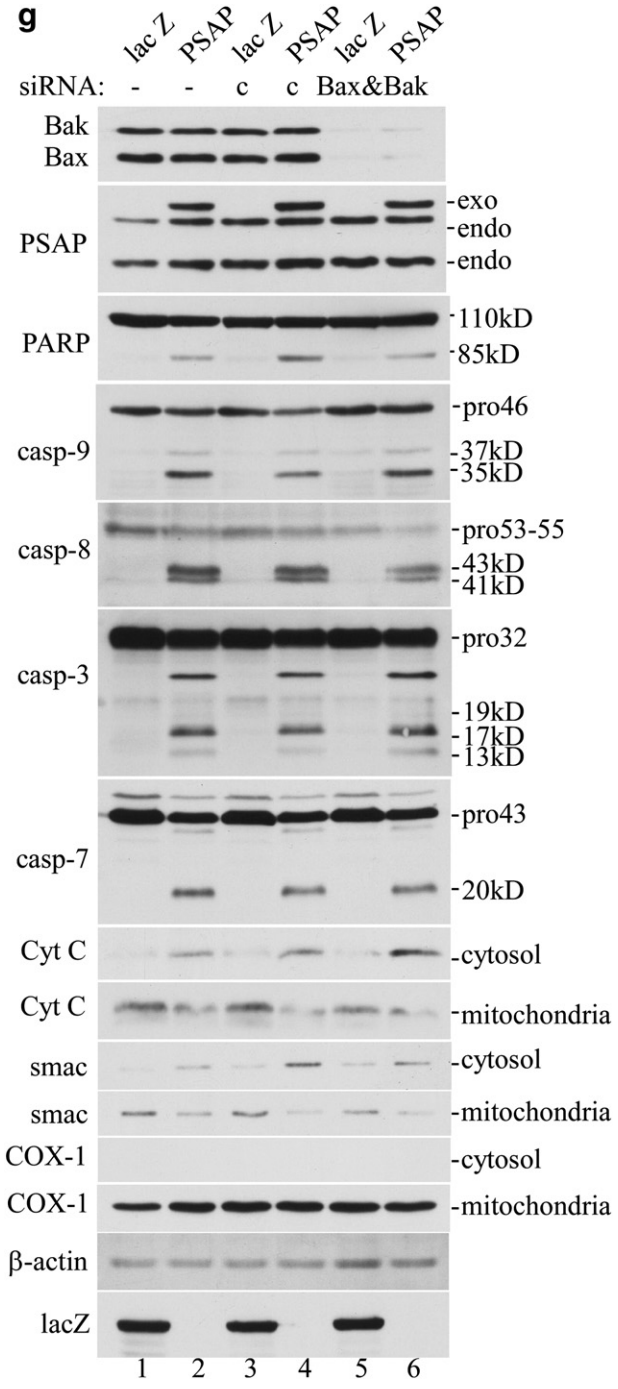
but had no effect on PSAP-induced release of cytochrome c and Smac. Mitochondrial outer membrane permeabilization and the release of intermembrane space proteins, such as cytochrome c, are generally thought to require the activation of Bak or Bax, the proapoptotic members of Bcl-2 family proteins [13]. In this regard it is notable that inhibition of caspase activation blocked PSAP-induced Bax translocation, but had no effect on cytochrome c release. This finding raised a question as to whether PSAP-induced cytochrome c release is controlled by Bcl-2 family proteins. Using the siRNA approach, our data clearly demonstrate that knockdown of Bid, Bax, or Bak individually, and knockdown of both Bax and Bak simultaneously had no effect on PSAP-induced cytochrome c release. These results strongly suggest that PSAP-induced release of cytochrome c is not controlled by these proapoptotic Bcl-2 proteins. Furthermore, our data clearly demonstrated that overexpression of Bcl-2 and Bcl-xL did not protect cells from PSAP-induced apoptosis. Taken together, these observations strongly suggest that PSAP-induced apoptosis is not regulated by Bcl-2 proteins. However, these proteins may contribute to PSAP-induced apoptosis via the caspase-8-tBid amplification loop.

As discussed above, there are two initiator caspases, caspase-8 and caspase-9, mediating the death receptor signaling pathway and mitochondrial pathway, respectively. In determining which caspase is the initiator caspase in PSAP-induced apoptosis, using the siRNA approach, our data demonstrated that knockdown of caspase-8 had no effect on PSAP-induced PARP cleavage (Fig. 4), nor caspase-3 and caspase-9 activation (Fig. 4). These data clearly argue against a role of caspase-8 in meditating the initiation of PSAP-induced apoptosis. Conversely, knockdown of caspase-9 completely abolished PSAP-induced PARP cleavage and the activation of caspase-3 and caspase-8, suggesting that caspase-9 is the initiator caspase in PSAP-induced apoptotic cascades. Caspase-9 is believed to be activated at the apoptosome, a complex formed by binding of cytochrome c to Apaf-1 in the presence of dATP in the cytosol [22]. To determine the role of the apoptosome in PSAP-induced apoptosis, we examined the effect of knockdown of Apaf-1 on PSAP-induced caspase-9 activation. The results that knockdown of Apaf-1 completely abolished activation of caspase-9 and other caspases, as well as the cleavage of PARP, clearly indicate that PSAP-induced caspase-9 activation is mediated by the Apaf-1-cytochrome c complex.

In addition to cytochrome c, Smac is another important proapoptotic factor released from mitochondria that promotes cytochrome c-Apaf-1-dependent caspase activation by neutralizing the inhibitory activity of XIAP, which is a member of the IAP family of proteins that block caspase activation [12]. Having shown that Smac was also released from mitochondria upon induction of PSAP expression, we wished to determine whether Smac also plays a role in PSAP-induced apoptosis. We therefore examined the effect of knockdown of Smac on PSAP-induced apoptosis. We found that knockdown of Smac completely abolished PSAP-induced PARP cleavage and caspase activation, indicating that, in addition to Apaf-1 or apoptosome formation, Smac is also required for PSAP-induced apoptosis. We also found that cytochrome c release was not significantly affected by knockdown of Smac, indicating that PSAP-induced cytochrome c release is independent of Smac.

PSAP shares homology within the mitochondrial carrier domain with mitochondrial carrier proteins [14]. Recently, a protein known as MTCH2, a close homologue of PSAP, was also reported to be involved in apoptosis [23]. Different from other mitochondrial carrier proteins, which are localized to the inner mitochondrial membrane [24], both PSAP and MTCH2 are apparently exposed on the surface of the mitochondria [17,25]. The biological functions of these two proteins remain unknown, but both are now implicated in apoptosis.



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However, MTCH2 itself does not cause apoptosis, but instead sensitizes cells to apoptotic stimuli by facilitating tBid recruitment through direct interaction with tBid [23]. In contrast, PSAP induces apoptosis when it is overexpressed and induces mitochondria dysfunction in a Bcl-2 protein-independent manner. Thus, PSAP and MTCH2 are involved in apoptosis via apparently different mechanisms. Accumulating evidence suggests that apoptosis is one of the mechanisms leading to neuronal cell death in Alzheimer's disease [26,27], and specifically, studies strongly suggest the involvement of dysfunction of mitochondria in neuronal degeneration in Alzheimer's disease [28,29]. Nevertheless, the molecular mechanism of mitochondrial dysfunction in Alzheimer's disease remains elusive. In this regard, as shown in Fig. 7, it is notable that PSAP induces release of cytochrome c and Smac/Diablo in a non-traditional manner, a Bcl-2 protein-independent manner, suggesting a new mitochondrial dysfunction pathway. This result may establish a new model for investigation of mitochondrial apoptosis observed in AD.

In summary, we have shown that PSAP-induced apoptosis requires both the formation of cytochrome c-Apaf-1 apoptosome and the presence of Smac. More interestingly, PSAP induces the release of the mitochondrial proapoptotic factors cytochrome c and Smac in a Bcl-2 protein-independent manner, and this may open a new avenue to study the mechanism of mitochondria-mediated apoptosis.

Acknowledgments

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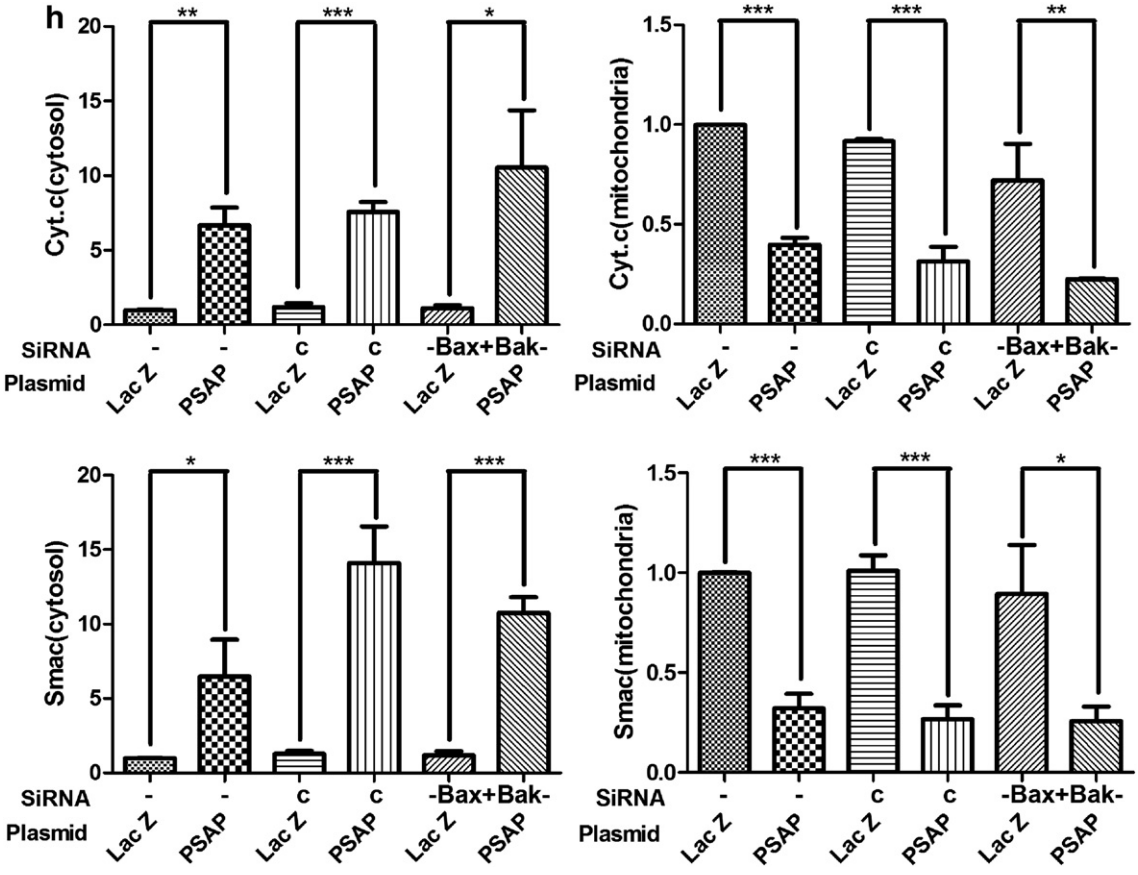

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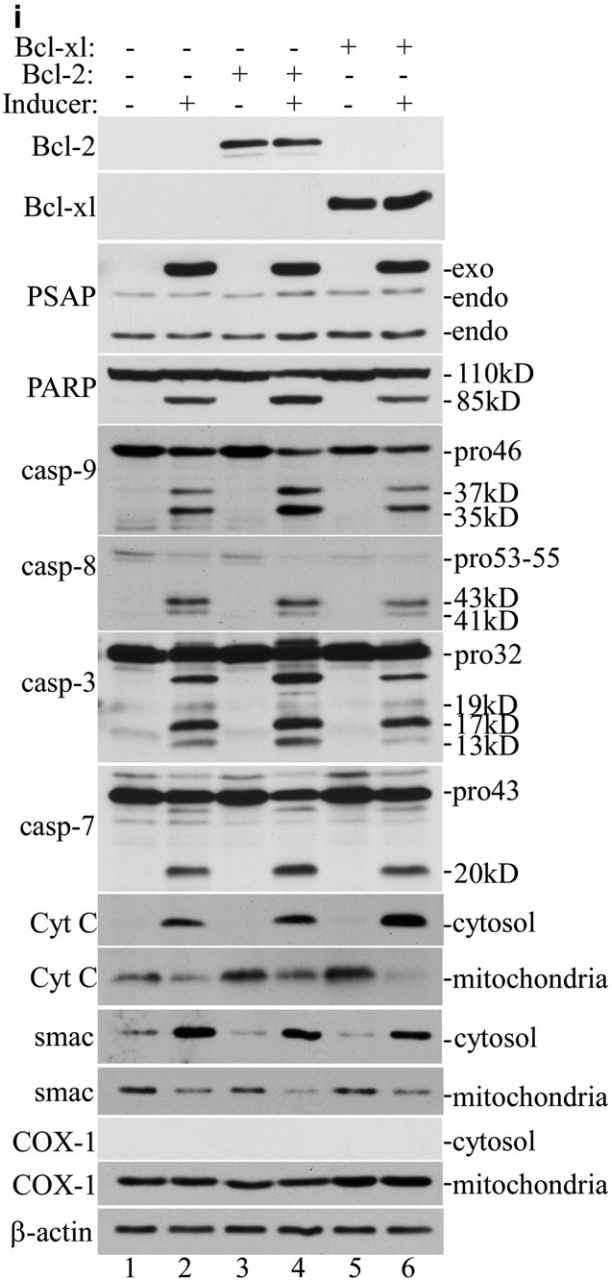

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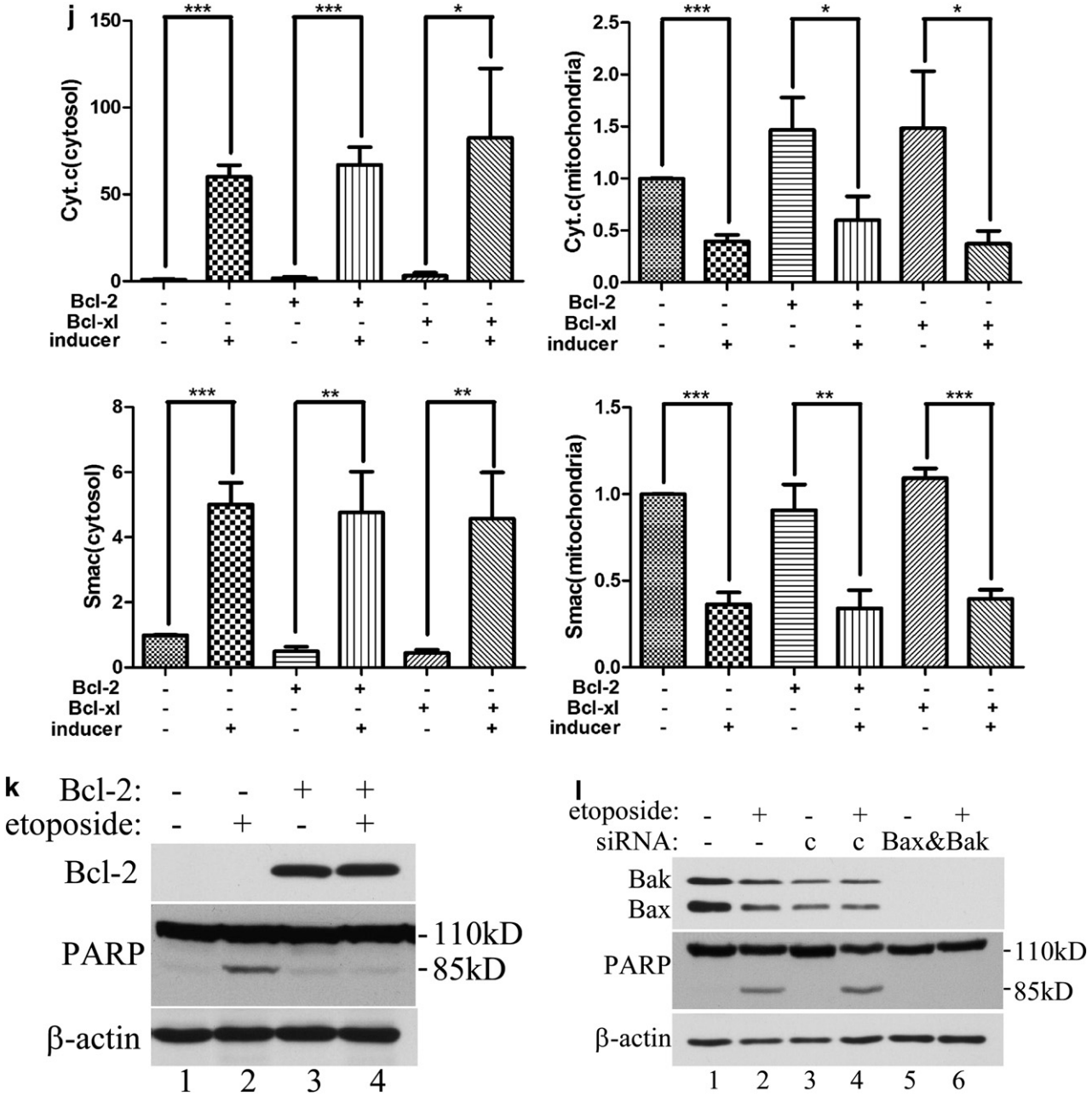

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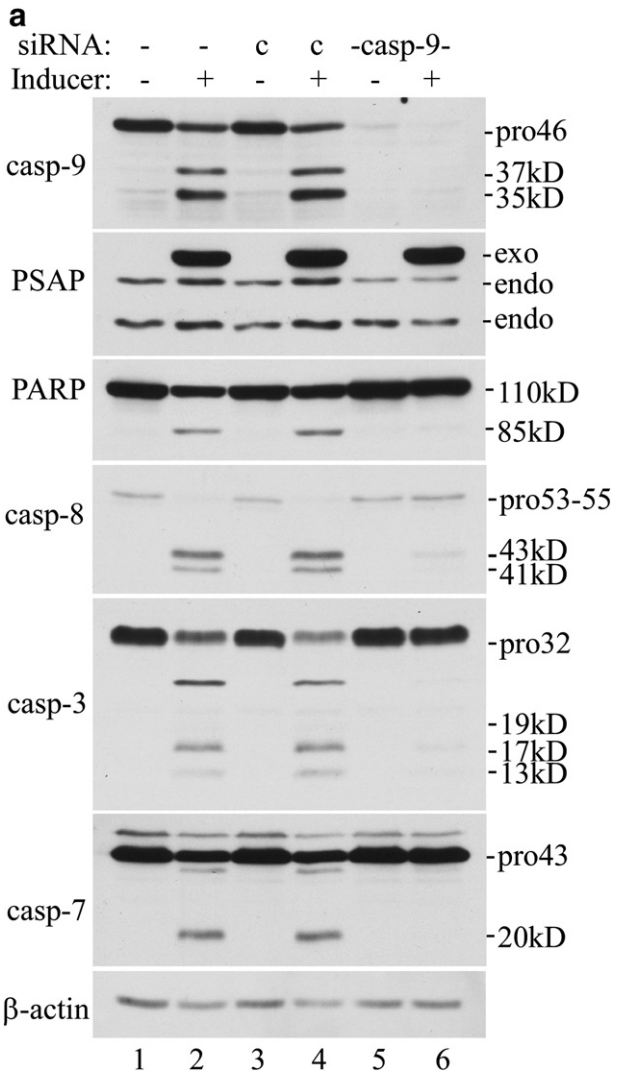
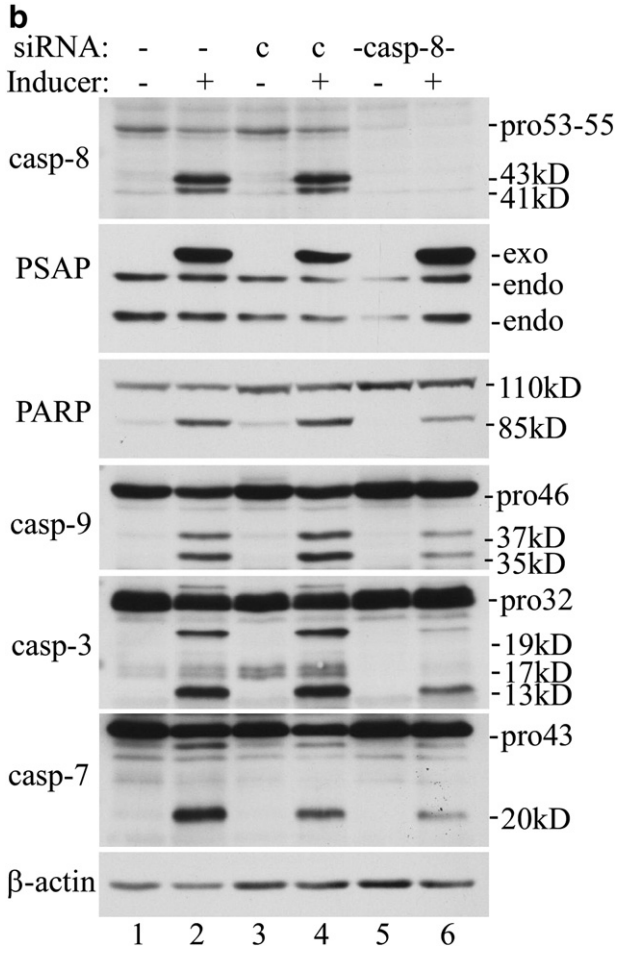


Fig. 4. Knockdown of caspase-9, but not caspase-8, abolished PSAP-induced apoptosis. Lanes 5 and 6 of top panels in a and b show complete knockdown of caspase-9 and caspase-8, respectively. Lanes 2, 4, and 6 of the second panels in A and B show the induced expression of PSAP. The third panels in A and B were probed for PARP cleavage. The fourth, fifth, and sixth panels in A were probed for caspase-8, -3, and -7, respectively. The fourth, fifth, and sixth panels in B were probed for caspase-9, -3, and -7, respectively. The membrane of the top panels in a and b were reprobed with anti-actin antibody to indicate relative loading of samples (bottom panel).



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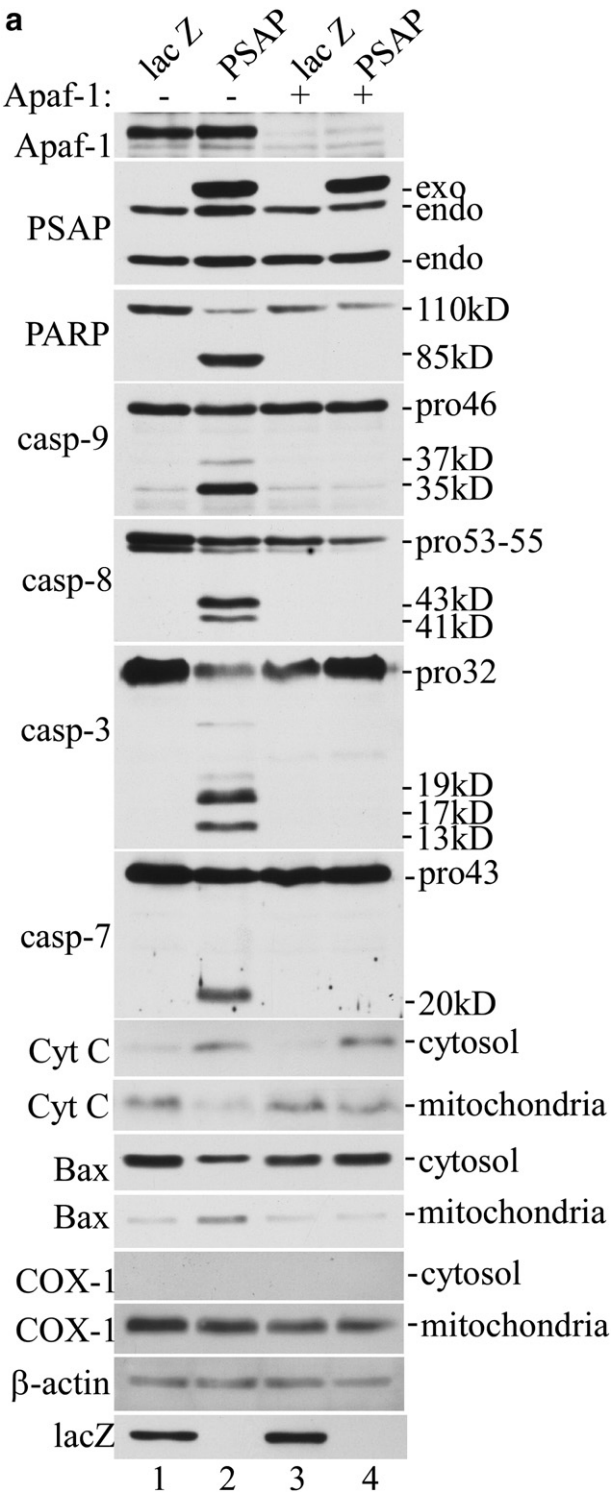

Fig. 5. Knockdown of Apaf-1 abolished PSAP-induced apoptosis, caspase activation, and Bax translocation, but not cytochrome c release. a. Cells stably expressing Apaf-1-specific shRNA were transfected with either LacZ-expressing plasmid (lanes 1 and 3) or PSAP-expressing plasmid (lanes 2 and 4). *Top panel*, complete knockdown of Apaf-1 (lanes 3 and 4). *Second panel*, immunoblot probed with anti-PSAP to determine the transient expression of PSAP (lanes 2 and 4). The bottom panel is the reprobe of the membrane in the second panel with anti-LacZ antibody to determine the transient expression of LacZ (lanes 1 and 3). *Third panel*, immunoblot probed with anti-PARP antibody to determine the cleavage of PARP upon expression of PSAP. *Fourth, fifth, sixth, and seventh panel*, immunoblots probed with specific antibodies to determine the activation of caspase-9, -8, -3, and -7, respectively. *Eighth and ninth panels,* immunoblots probed for cytochrome c in cytosolic (eighth panel) and mitochondrial (ninth panel) fractions, respectively. *Tenth and eleventh panels*, immunoblots probed for Bax in cytosolic (tenth panel) and mitochondrial (eleventh panel) fractions, respectively. *Twelfth and thirteenth panels*, immunoblots probed for COX-1 in cytosolic (twelfth panel) and mitochondrial (thirteenth panel) fractions, respectively. The membrane of the second panel was reprobed with anti-actin antibody to indicate relative loading of samples (fourteenth panel). b. Quantification and statistical analysis of the Western blot results for cytochrome c release and Bax translocation shown in a. Single, double, and three asterisks indicate significant differences between control and treatment at $p<0.05$ 0.01, and 0.001 respectively.

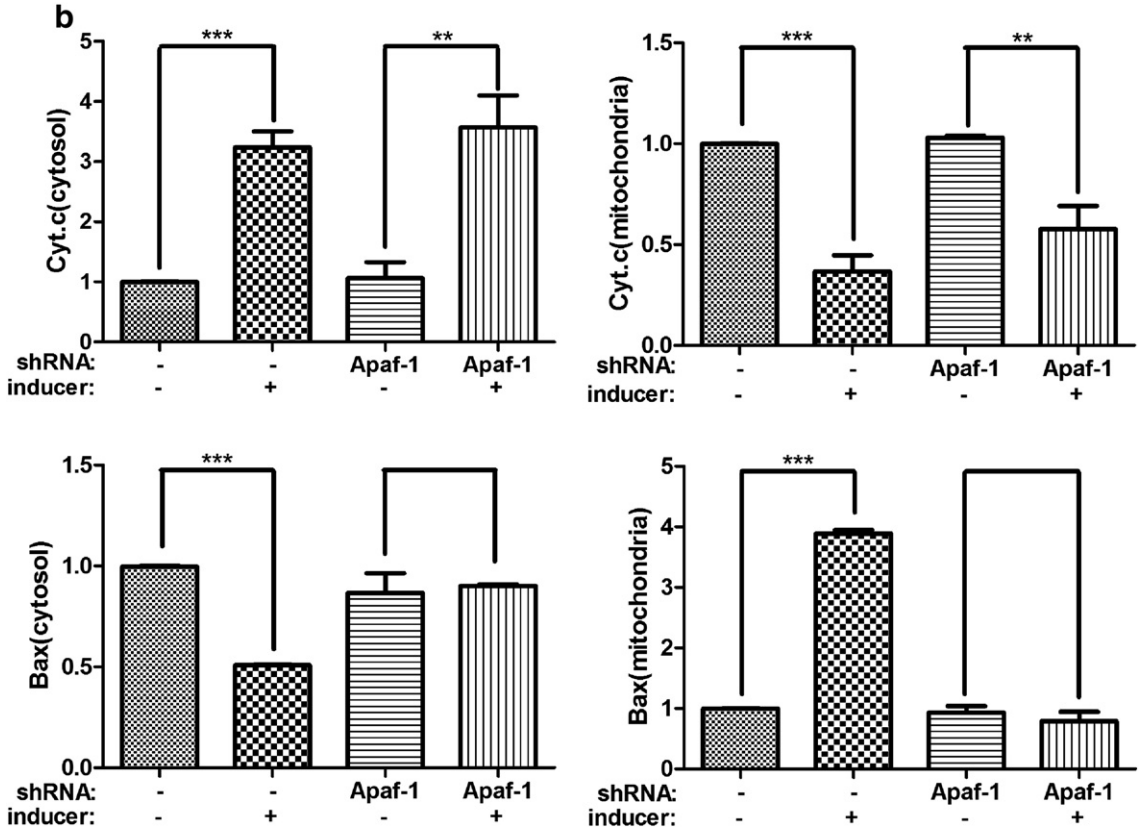

Fig. 5 (*continued*).

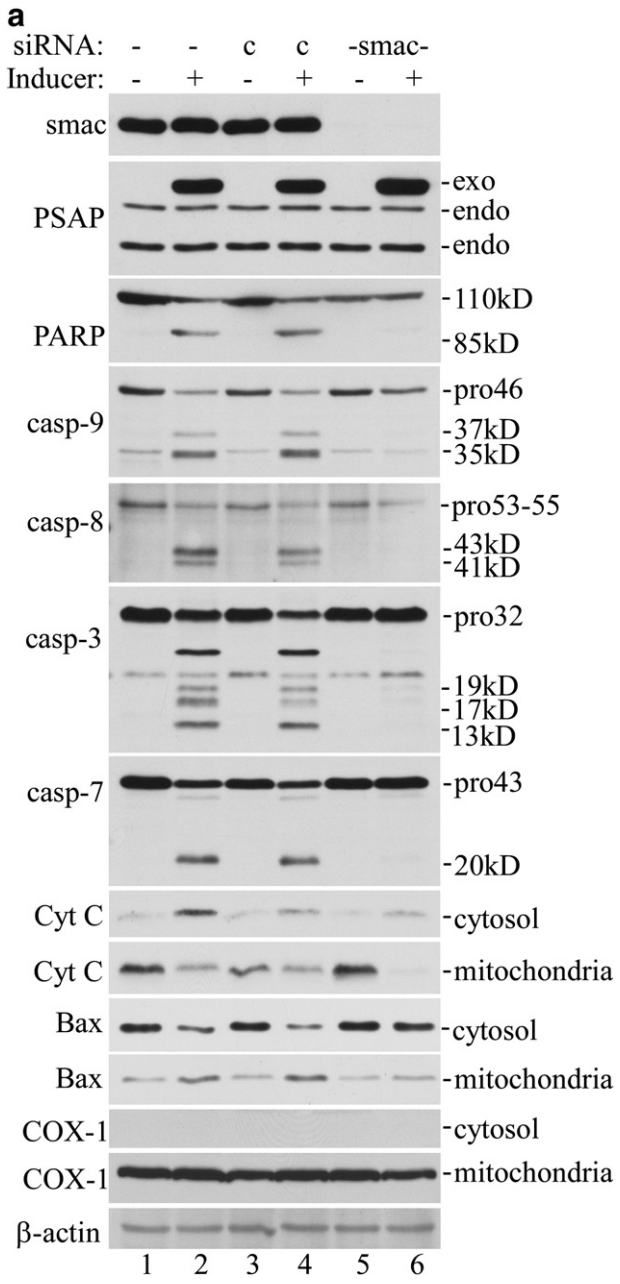

Fig. 6. Knockdown of Smac abolished PSAP-induced apoptosis, caspase activation, and Bax translocation, but not cytochrome c release. **a**. Cells expressing inducible PSAP were treated with transfection reagent (lanes 1 and 2), control non-silencing siRNA (lanes 3 and 4), and siRNA-specific to Smac (lanes 5 and 6). Complete knockdown of Smac was determined in Smac-siRNA treated cells (top panel, lanes 5 and 6). *Second panel*, immunoblot probed with anti-PSAP to determine the inducible expression of PSAP (lanes 2, 4, and 6). *Third panel*, immunoblot probed with anti-PARP antibody to determine the cleavage of PARP upon induction of PSAP expression. *Fourth, fifth, sixth, and seventh panels*, immunoblots probed with specific antibodies to determine the activation of caspase-9, -8, -3, and -7, respectively. *Eighth and ninth panels*, immunoblots probed for cytochrome c in cytosolic (eighth panel) and mitochondrial (ninth panel) fractions, respectively. *Tenth and eleventh panels*, immunoblots probed for Bax in cytosolic (tenth panel) and mitochondrial (eleventh panel) fractions, respectively. *Twelfth and thirteenth panels*, immunoblots probed for COX-1 in cytosolic (twelfth panel) and mitochondrial (thirteenth panel) fractions, respectively. The membrane of the second panel was reprobed with anti-actin antibody to indicate relative loading of samples (fourteenth panel). b. Quantification and statistical analysis of the Western blot results for cytochrome c release and Bax translocation shown in a. Single, double, and three asterisks indicate significant differences between control and treatment at $p<0.05$ 0.01, and 0.001 respectively.

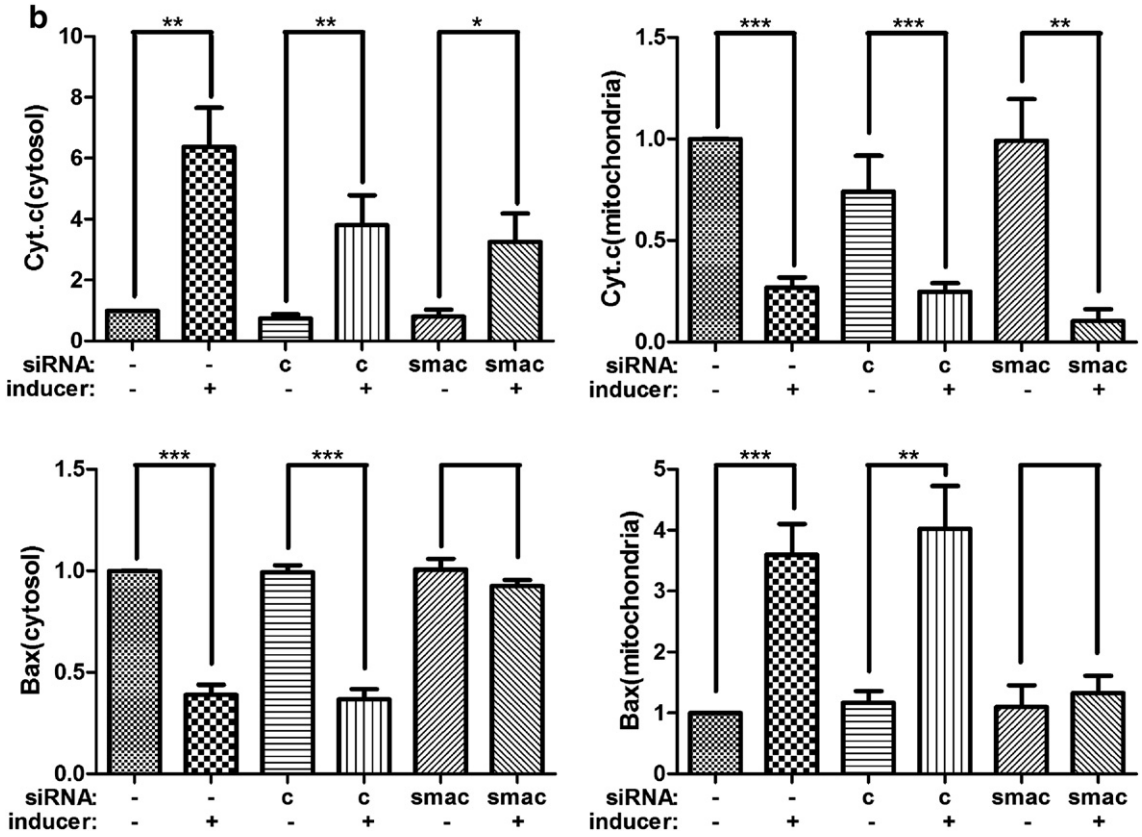

Fig. 6 (*continued*).

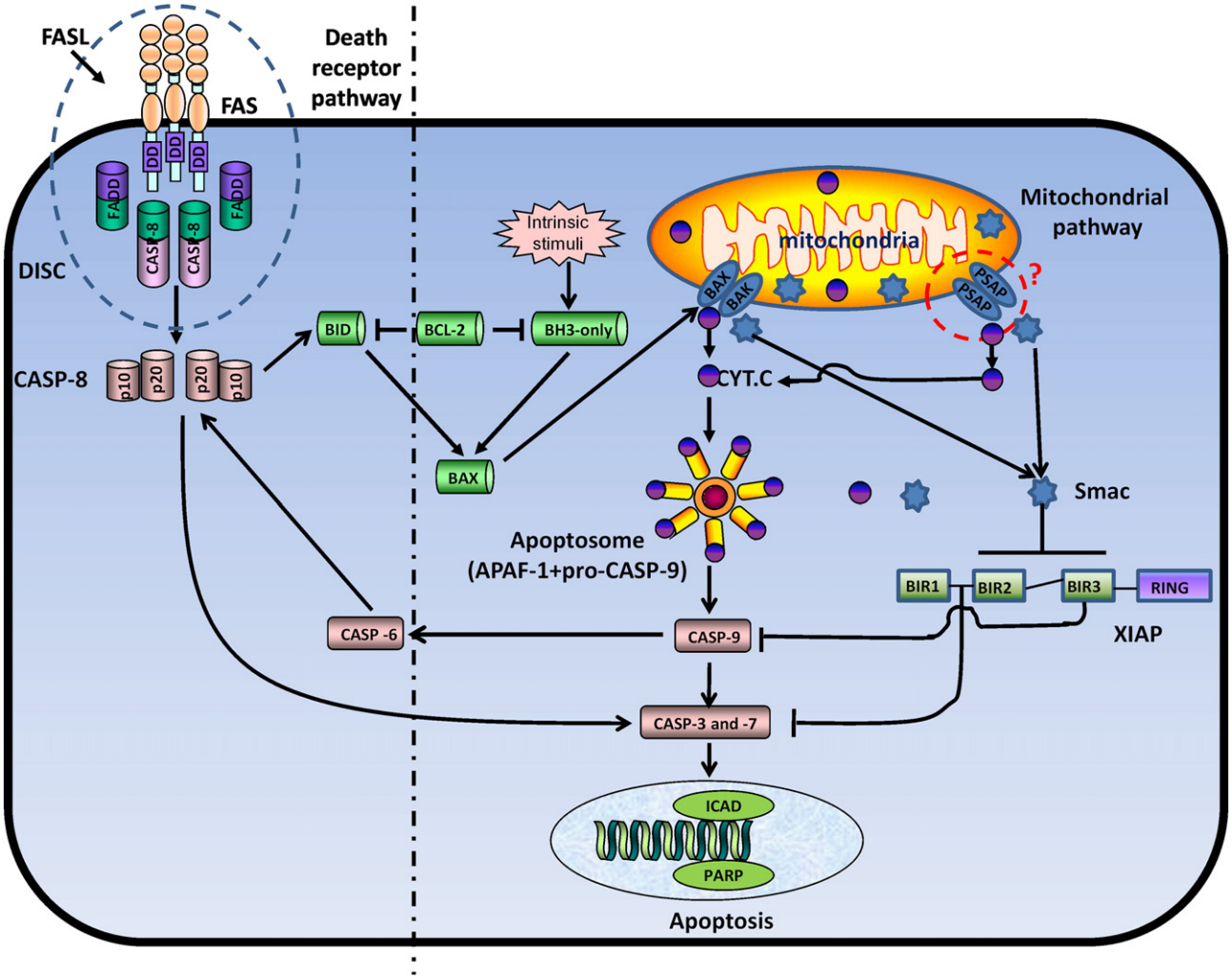

Fig. 7. Schematic diagram of death receptor (DR) and mitochondrial pathways for the induction of apoptosis. In the extrinsic DR pathway, ligation of DR (for example, FAS–FASL) results in the formation of DISC leading to the activation of caspase-8, which might lead directly to the activation of caspase-3 or caspase 7, which in turn cleaves intracellular substrates, resulting in apoptosis. In the traditional intrinsic mitochondrial pathway, intracellular death signals, such as DNA damage and other cellular stress signals, are transmitted to the mitochondria through pro-apoptotic Bcl-2 family proteins, such as Bid and Bax. Activated Bid (tBid) stimulates Bax to translocate to the outer mitochondrial membrane where it forms a complex with Bak resulting in the release of cytochrome c (purple), Smac/DIABLO (blue), and other apoptogens (not shown). The anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-xL (not shown), inhibit this process through interacting with the pro-apoptotic proteins. For review, see [13,30]. However, the mitochondrial pro-apoptotic protein PSAP induces release of cytochrome c and Smac/DIABLO in a Bcl-2 protein-independent manner, suggesting a new mitochondrial apoptotic pathway. XIAP (X-linked IAP) is an IAP, which binds and inhibits caspase activity. These caspase inhibitors can be antagonized by the pro-apoptotic protein Smac/DIABLO [31]. Inhibitor of caspase-3-activated DNase (ICAD) and poly (ADP-ribose) polymerase (PARP) are substrates of caspases.