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Apoptosis Induced by a New Member of Saponin Family is Mediated through Caspase-8 Dependent Cleavage of Bcl-2

Jianbei Zhu, Lei Xiong, Biao Yu and Jiarui Wu

Laboratory of Proteomics, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, China (J.Z., L.X., J.W); State Key Laboratory of Bio-organic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 354 Feng-Lin Road, Shanghai 200032, China (B.Y.); Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science & Technology of China, Hefei, Anhui 230027, China (J.W).

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Running title: Apoptosis by OSW-1 results from Bcl-2 cleavage

Corresponding Author: Jiarui Wu

Institute of Biochemistry and Cell Biology

Shanghai Institutes for Biological Sciences, CAS

320 Yue-Yang Road.

Shanghai 200031

China

Fax: 021-54921011

Email: wujr@sibs.ac.cn

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Abbreviations: CHO, Chinese hamster ovary; EGFP, enhanced green fluorescent protein; FACS, fluorescence activated cell sorter; FADD, Fas-associating death domain-containing protein; SDS-PAGE, SDS polyacrylamide gel electrophoresis; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

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Abstract

OSW-1 is a new member of cholestane saponin family, which is cytotoxic against several types of malignant cells. We reported herein that OSW-1 induced apoptosis of mammalian cells in a concentration- and time-dependent manner. The drug-induced apoptosis was mediated through the mitochondrial pathway, involving the cleavage of Bcl-2. This drug-induced Bcl-2 cleavage in CHO cells could be suppressed either by dominant negative caspase-8 or by a caspase-8 inhibitor, suggesting that the Bcl-2 cleavage is dependent on caspase-8. In contrast, the Bcl-2 cleavage was independent of caspase-3 activity. The inhibition of caspase-8 activity also resulted in the reduction of apoptotic cells, indicating that Bcl-2 cleavage induced by caspase-8 promotes the progression of apoptosis. The involvement of the caspase-8 activity in the processes of the OSW-1 induced apoptosis was further examined by using caspase-8 deficient Jurkat T cells. It was found that the caspase-8 deficient cells were resistant to OSW-1 induced Bcl-2 cleavage or apoptosis. Furthermore, the small subunit of caspase-8 was found to interact with Bcl-2 as determined by yeast two-hybrid and co-immunoprecipitation assays. Overexpression of caspase-8 small subunit reduced the cleavage of Bcl-2 and inhibited the apoptosis induced by OSW-1. Taken together, these results demonstrate that OSW-1 is capable of inducing apoptosis in mammalian cells, in which the caspase-8 dependent cleavage of Bcl-2 plays an important role.

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Introduction

Saponins belong to a family of glycoconjugtes with a broad spectrum of biological and pharmacological activities (Hostettmann and Marson, 1995). As a new member of cholestane saponin family, OSW-1, namely 3β, 16β, 17α-trihydroxycholest-5-en-22-one 16-*O*-{*O*-(2-*O*-(4-methoxybenzoyl)-β-D-xylopyranosyl)-(1→3)-2-*O*-acetyl-α-arabinopyranoside}, was first isolated from the bulbs of *Ornithogalum saudersiae* (Kubo et al., 1992). Its total-chemical-synthesis was subsequently accomplished (Deng et al., 1999). It has been reported that OSW-1 was cytotoxic against several types of malignant cells at nanomolar concentrations, which are about 10-100 times more potent than those of the clinically applied anticancer agents including mitomycin C, adriamycin (Mimaki et al., 1997). Despite its highly potent antitumor activity and unique chemical structure, the molecular basis of its mechanism of action has remained elusive.

Apoptosis is a universal cellular process, which plays an important role in normal development as well as pathology of a number of human diseases. The resistance to apoptosis is a general feature of cancer cells. There are two main pathways involved in apoptosis. The extrinsic apoptotic pathway is activated by the ligation of death receptors, whereas the intrinsic apoptotic pathway is mediated through mitochondria (Zimmermann et al., 2001). The death receptors such as Fas recruit the adaptor protein FADD, which in turn recruits the proform of caspase-8. Aggregation of procaspase-8 leads to its auto-activation and subsequent activation of executioner caspases (Thorburn, 2004). The apoptotic signal can also be amplified through the mitochondria by altering its membrane permeability to facilitate the release of apoptogenic proteins like cytochrome *c*, which is regulated by the members of Bcl-2 family (Degli Esposti, 2004).

Bcl-2 family proteins are subdivided into either anti-apoptotic members such as Bcl-2 and

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Bcl-X_L, which inhibited the cytochrome *c* release from mitochondria or pro-apoptotic members such as Bax and Bak, which promote the release of cytochrome c (Yang et al., 1997; Marzo et al., 1998). Overexpression of Bcl-2 prevents cells from undergoing apoptosis due to its ability of preserving the mitochondrial membrane integrity (Yang et al., 1997). It has been shown that Bcl-2 is cleaved by caspases during apoptosis, which results in the inactivation of Bcl-2 (Grandgiard et al., 1998), or even converts Bcl-2 to a Bax-like fragment (Cheng et al., 1997). The inhibition of caspase-induced Bcl-2 cleavage results in the suppression of apoptosis (Kim et al., 1998). Although some experiments showed that caspase-3 was involved in the Bcl-2 cleavage (Grandgiard et al., 1998; Cheng et al., 1997; Zhang et al., 1999), the importance of caspase-3 in this process remains unclear as Bcl-2 cleavage has been shown to occur in caspase-3 deficient MCF-7 cells (Kim et al., 1998).

Bcl-2 family members may facilitate crosstalk between the death receptor and mitochondrial pathways. For example, the cleavage of Bid, a "BH3-domain-only" Bcl-2 family member, by caspase-8 activates the mitochondrial pathway in apoptosis induced by death receptors (Li et al., 1998). Some data suggest that caspase-8 is related to the regulation of the inhibitory effect of Bcl-2 on apoptosis. The inhibition of caspase activation by Bcl-2 could be overcome by adding active caspase-8 in the *Xenopus* cell-free system (Kuwana et al., 1998), whereas Bcl-2 could inhibit TRAIL-induced activation of caspase-8 and cell death (Fulda et al., 2002). In this manuscript, we show that the cleavage of Bcl-2 induced by OSW-1 in mammalian cells is mediated by caspase-8 rather than by caspase-3, while this Bcl-2 cleavage might promote the progress of apoptosis.

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Materials and Methods

Cell Culture. Chinese hamster ovary (CHO) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 100 μM non-essential amino acids (Gibco BRL, Grand Island, NY). FADD and caspase-8-deficient Jurkat T cells (Juo et al., 1998; Juo et al., 1999) as well as their corresponding parental cell line A3 were kindly provided by Dr. Junying Yuan of Harvard Medical School, maintained in RPMI-1640 medium supplemented with 10% FBS.

Plasmid Constructions. The pEGFP-bcl-2 encoding EGFP-Bcl-2 fusion protein under the control of cytomegalovirus promoter was constructed by inserting full-length human bcl-2 cDNA into pEGFP-C vector (Clontech, Palo Alto, CA, USA). A pEGFP-C vector containing catalytically inactive mutant caspase-3 gene was constructed by introducing into a point mutation that substituted serine for the active site cystine-163 of caspase-3. The following mutagenesis primers were used to generate caspase-3 mutant: 5'-forward (5' CATTATTCAGGCCTCCCGTGGTACAG 3') and 3' –reverse (5' CTGTACCACGGGAGGCCTGAATAATG 3'). In addition, the pFLAG-caspase-8 dominant negative plasmid was a gift from Dr. Teshiyuki Miyashita (U et al., 2001). pEGFP-casp8p10 was generated by inserting caspase-8 small subunit p10 sequence into pEGFP-C vector.

Stable Transfections. CHOC 400 cells were transfected with pEGFP-bcl-2 and CHO AA8 cells were transfected with pFLAG-caspase-8 dominant negative plasmid by lipofectamine^{TM} 2000 system (Gibco BRL, Grand Island, NY). The cells were selected in the presence of G418 in DMEM medium for approximately 20 days. After the selection, Bcl-2 expression level of individual clones was determined by the detection of EGFP expression level with flow cytometer (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA), while the FLAG-caspase-8 expression

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was determined by Western blotting with an anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA).

Transient Transfections. CHO AA8 cells were transiently transfected with mutant caspase-3 plasmid and pEGFP-casp8p10 plasmid, respectively, by electroporation with Nucleofector T kit according to the manufacturer's instructions (Amaxa Biosystems, Cologne, Germany). The overexpression of mutant caspase-3 or p10 of caspase-8 was verified either by the detection of EGFP expression level on flow cytometer or by Western blotting assay. The transfection efficiency was up to 70%.

Preparation of Cytosolic and Mitochondrial Extracts by Digitonin Treatment. CHO AA8 cells were harvested and resuspended in a buffer (20 mM Hepes-KOH, pH 7.3, 110 mM KAc, 5 mM NaAc, 2 mM $MgAc_2$, 1 mM EGTA) containing 200 μg/ml digitonin (Calbiochem-Novabiochem, La Jolla, CA, USA) on ice for 10 minutes. The permeabilized cells containing cellular organelles and nuclei were pelleted by centrifugation as mitochondrion-fractions, and the supernatants were collected as cytosolic fractions.

Western Blotting Analysis. Intact cells, supernatants and the pellets of digitonin-treated cells were added with loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol and 0.1% bromophenol blue). The equalized amounts of proteins from each sample were subjected to SDS-PAGE. Western blotting was carried out with primary antibodies (anti-Bax, anti-cytochrome *c* and anti-GFP from Santa Cruz Biotechnology, CA, USA; anti-Bcl-2 (human) and anti-Bcl-2 (hamster) from Sigma-Aldrich, St. Louis, MO, USA; anti-caspase-8 and anti-FADD from BD Biosciences, San Diego, CA, USA; anti-caspase-3 from Cell Signaling Technology, Beverly, MA, USA), followed by horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, USA). Immune complexes were detected by the enhanced

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chemiluminescence's system according to the manufacturer's instructions (ECL, Amersham Biosciences, Little Chalfont, UK).

Immunoprecipitation Analysis. CHO AA8 cells stably transfected with the pEGFP-casp8p10 or the vector were lysed in the cell lysis buffer (50 mM Tris-HCl, pH 7.6, 0.5% Triton X-100, 5 mM EDTA, 1 mM Na_3VO_4, 2 μg/ml leupeptin, 2 μg/ml antipain, 20 μg/ml benzamidine, 2 μg/ml chymostatin, 2 μg/ml pepstatin, 1 mM PMSF) and ultrasonicated for 2 minutes on ice. The mixtures were centrifugated and the supernatants were incubated first with anti-Bcl-2 antibody at 4 ^{0}C for 3 hr, and then incubated with protein G-agarose (Santa Cruz Biotechnology, CA, USA) overnight. The washed immunoprecipitates were subjected to immunoblotted analysis with anti-GFP and anti-Bcl-2 antibodies.

Flow Cytometric Analysis. In order to identify sub-G1 DNA region (below the G0/G1 peak), which is indicative of cells undergoing apoptosis, the drug-treated cells were harvested and fixed with 70% ethanol. The fixed cells were stained with propidium iodide and analyzed by the flow cytometer (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometric analysis with Annexin V-FITC was done according to the manufacturer's instructions (BD PharMingen, San Diego, CA, USA).

DNA Fragmentation Assay. DNA of CHO AA8 cells was prepared as described by Hockenbery et al. (1990) Briefly, the drug-treated cells were lysed in a cell lysis buffer (10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.25% Triton X-100) on ice for 30 minutes. After centrifugation of the cell lysates, the supernatants were incubated with 100 μg/ml RNase at 37 ^{0}C for 30 minutes, and then with 200 μg/ml proteinase K at 56 ^{0}C overnight. The mixture was extracted with phenol-chloroform and precipitated with ethanol. The pellets were resuspended in Tris-EDTA buffer and subjected to agarose gel electrophoresis.

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Caspase-8 Activity Assay. CHO cells were treated with OSW-1 for indicated time. Then the caspase-8 activities were monitored using the caspase-8 activity assay kit according to the manufacturer's protocol (Calbiochem-Novabiochem, La Jolla, CA, USA).

Yeast Two-hybrid Assay. The yeast two-hybrid assay for detecting interaction between caspase-8 and Bcl-2 was carried out basically according to the strategies from the reference Kamada and Tsujimoto (2000), in which both large and small subunits of caspase-8 separately expressed in yeast can be kept in equimolar ratio of large to small subunits. Briefly, fragments encoding caspase-8 subunits or Bcl-2 protein were generated by PCR and cloned into pGAD10, pGAD10□AD (pGAD10 lacking the Gal4 activation domain) or pBTM116, to get pGAD10-bcl-2, pGAD10□AD-casp8-p18, pBTM-casp8-p10 and pBTM-casp8-p18. The fragment bearing casp8-p18 under control of ADH1 promoter from pGAD10□AD-casp8-p18 was cloned into the PvuII site of pBTM-casp8-p10 to generate pBTM-casp8-p10p18. $p18^{m}$ was constructed by introducing a point mutation (Cys→Ser), which is the same as the mutation in the pFLAG-caspase-8 dominant negative plasmid, into the p18 fragment. The bait plasmids pBTM-casp8-p10p18, pBTM-casp8-p10 or pBTM-casp8-p18 were cotransformed into yeast strain L40 (MAT a trp1 leu2 his3 ade2 LYS::LexA-His3 URA3::LexA-lacZ) with the prey plasmid pGAD10-bcl-2 by LiAc yeast transformation assay. β-galactosidase activity was detected by the filter lift assay according to the Yeast Protocols Handbook (Clontech). The plasmids pBTM116 and pGAD10, and the yeast strain L40 were gifts from Dr Youshihide Tsujimoto.

Statistical Analysis. All the data in this study were expressed as the mean±standard deviation (SD) from at least three independent experiments. Statistical analysis was performed using one-way ANOVA or independent-samples T test. A value of $p<0.05$ was considered statistically significant.

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Results

OSW-1 Induces Apoptosis in CHO Cells in a Dose- and Time-dependent manner. OSW-1 has been shown to possess potent antitumor activity (Mimaki et al., 1997). In order to analyze its biological effects, exponentially growing CHO AA8 cells were treated with 200 ng/ml synthetic OSW-1 for 24 hr. Apoptosis of the drug-treated cells was measured by three apoptotic assays, including sub-G1 DNA content analysis, Annexin-V assay and DNA fragmentation assay (Sgonc and Gruber, 1998; Vermes et al., 1995; Zhang and Xu, 2000) (Fig. 1*A*). Significant apoptosis was seen by all three assays. Further analysis showed that OSW-1 induced apoptosis in CHO cells in a dose- and time-dependent manner (Fig. 1*B*).

OSW-1-induced Apoptosis is Mediated through Mitochondrial Pathway. In order to address the molecular mechanisms of the OSW-1 induced apoptosis, we monitored the changes of apoptotic molecules related to mitochondrial pathway in OSW-1 treated CHO cells. After drug treatment, the floating cells taken as the entire apoptotic population were collected and fractionated into the cytosolic fractions and mitochondria-containing fractions by digitonin-permeabilization assay (see Materials and methods). Western blotting analysis showed that Bcl-2 in OSW-1-treated cells was significantly cleaved into a fragment about 23 Kd (□Bcl-2) (Fig. 2*A*, compare lanes 1 and 3 to 4 and 6). In addition, Bax proteins were translocated from cytosol to mitochondria in drug-treated cells, whereas cytochrome *c* molecules were released from mitochondria to cytosol (Fig. 2*A*, compare lanes 2 and 3 to 5 and 6). Cox 4 (cytochrome *c* oxidase IV), as a control, remained in the mitochondrion fractions (Fig. 2*A*, lanes 3 and 6). These results indicate that the OSW-1-induced apoptosis is likely to be mediated through the mitochondrial pathway.

It has been reported that the cleavage of Bcl-2 by caspases promotes the progression of

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apoptosis (Cheng et al., 1997; Grandgiard et al., 1998). To further investigate the effect of Bcl-2 cleavage on the OSW-1 induced apoptosis, we generated a stably transfected CHOC 400 cell line that overexpresses human Bcl-2 protein. It was shown that the exogenous Bcl-2 did locate in the mitochondrial membrane (Fig. 2*B*, lane 6), suggesting it could function as the normal endogenous Bcl-2 proteins. OSW-1 induced cleavage of Bcl-2 was inhibited in the cells overexpressing Bcl-2 proteins, whereas the significant degradation of Bcl-2 was detected in the control cells (Fig. 2*C*, compare lanes 4 and 6 to 10 and 12). Furthermore, the translocation of Bax from cytosol to mitochondria and the release of cytochrome *c* from mitochondria to cytosol were also blocked in Bcl-2-overexpressed cells (Fig. 2*C*, compare lanes 5 and 6 to11 and 12). In addition, the CHO cells overexpressing Bcl-2 protein became resistant to the drug-induced apoptosis (Fig. 2*D*). Taken together, these results suggest that Bcl-2 cleavage induced by OSW-1 is required for the drug-induced apoptosis.

The Cleavage of Bcl-2 is Mediated by Caspase-8 rather than by Caspase-3. Since some previous experiments indicated that caspase-3 was involved in the cleavage of Bcl-2 (Grandgiard et al., 1998; Cheng et al., 1997; Zhang et al., 1999), the relationship between caspase-3 activity and Bcl-2 cleavage was examined in the context of OSW-1 induced apoptosis. The time course of activation of caspase-3 was determined by Western blotting analysis, in which an active form of caspase-3 should be cleaved to yield a p19 fragment (Li et al., 2002). The p19 fragment of caspase-3 was detected when the cells were treated with OSW-1 for more than 22 hours (Fig. 3*A*, upper panel). In contrast, the cleavage of Bcl-2 could be detected as early as 18 hours after drug treatment (Fig. 3*A*, lower panel). These results indicate that the time course of activation of caspase-3 is different from that of the Bcl-2 cleavage.

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To further investigate the role of caspase-3 in Bcl-2 cleavage, a caspase-3 selective inhibitor, Z-DQMD-fmk (Chou et al., 2004), was administered to the cells treated with OSW-1. The inhibition of the caspase-3 activity by DQMD-fmk did not prevent the cleavage of Bcl-2 (Fig. 3*B*, upper panel). The inhibition of casapse-3 activity was confirmed by the observation that the p19 fragment of caspase-3 was replaced by a p20 fragment (Fig. 3*B*, lower panel), which represents an inactive middle product of caspase-3 (Li et al., 2002). Furthermore, CHO cells overexpressing dominant negative caspase-3 were generated by substituting the catalytically active site cysteine with serine, which specifically blocked the activation of endogenous caspase-3 (Aouad et al., 2004). As shown in Figure 3*C* , OSW-1 induced Bcl-2 cleavage was detected in the cells either transfected with the vector or with the catalytically mutant caspase-3 plasmid. These results support the notion that OSW-1 induced Bcl-2 cleavage is independent of the caspase-3 activity.

Since it has been reported that caspase-8 is activated before the activation of caspase-3 (Scheel-Toellner et al., 2004), we next examined the relationship between caspase-8 activity and Bcl-2 cleavage. A widely used caspase-8 inhibitor, CP-IETD-cho (Suen et al., 2003), was added to cells treated with OSW-1. OSW-1-induced cleavage of Bcl-2 was inhibited by IETD-cho (Fig. 4*A*, right panel), when the activity of caspase-8 was inhibited as expected(Fig. 4*A*, left panel). To confirm this result, a CHO cell line stably transfected with a caspase-8 dominant negative plasmid was established (see Materials and Methods). OSW-1 induced Bcl-2 cleavage was undetectable in the cells overexpressing the catalytically inactive mutant of caspase-8 (Fig. 4*B*), consistent with the observation that inhibition of caspase-8 activity prevents the Bcl-2 cleavage. In addition, the inhibition of caspase-8 activity either by the inhibitor or by the dominant negative caspase-8 mutant did reduce the amount of apoptotic cells under OSW-1 treatment (Fig. 4*C*), suggesting that caspase-8 activity is required for the OSW-1 induced apoptosis, at least

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partially, by cleaving Bcl-2 molecules.

Caspase-8 Null Cells are Resistant to the OSW-1-induced Apoptosis. If capase-8 is involved in the OSW-1 induced apoptosis, the deficiency of caspase-8 should lead to the resistance of the cells to the drug-induced apoptosis. Thus, Jurkat T cells deficient in caspase-8 was treated with OSW-1. The wild-type A3 cells were employed as a positive control and Jurkat T cells deficient in FADD were used as a negative control, which are known to be resistant to death receptor-mediated apoptosis (Juo et al., 1998; Juo et al., 1999). Caspase-8 deficient Jurkat T cells treated with OSW-1 failed to undergo apoptosis ($p>0.05$, Fig. 5*B*). In contrast, both wild-type A3 cells and FADD deficient Jurkat T cells underwent apoptosis upon treatment with OSW-1 (Fig. 5*B*), although the apoptotic population of A3 cells was much higher than that of the FADD deficient cells (Fig. 5*B*). It has been shown that the Bcl-2 could be cleaved in Jukat T cells although the Bcl-2 cleavage was not significant, consistent with the previous observation (Shim JH et al., 2002). In the present study, our results showed that the Bcl-2 cleavage was detectable in wild-type cells and inconspicuous in FADD-/- cells (Fig. 5*A*), whereas no Bcl-2 cleavage was detected in caspase-8-/- cells (Fig. 5*A*). Taken together, these results suggest that the caspase-8 deficient cells are resistant to OSW-1 induced Bcl-2 cleavage or apoptosis.

There is Physical and Functional Interactions between Caspase-8 and Bcl-2. The results of the caspase-8 dependent cleavage of Bcl-2 indicated that Bcl-2 might be a proteolytic substrate of caspase-8, and thus these two proteins might have physical interactions with each other. To test this speculation, a yeast two-hybrid system was developed basically according to the strategies by Kamada and Tsujimoto, 2000 (see Materials and Methods). It was shown that either a bait plasmid containing both large and small subunits (p10p18) of active caspase-8 or only the small subunit (p10) resulted in the blue-color reaction for β-galactosidase filter assay

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(Fig. 6*A*), indicating that caspase-8 is capable of interacting with Bcl-2 via its small subunit. To further confirm this observation, a point mutation was introduced into active site cysteine of p18, which prevents the proteolytic cleavage of the substrate and results in the formation of a stable enzyme-substrate complex in yeast cells (Kamada and Tsujimoto, 2000). Indeed, the bait plasmid containing both the small subunit and mutated large subunit ($p10p18^{m}$) resulted in higher β-Gal activity, indicative of stronger interactions (Fig. 6*A*). Furthermore, the co-immunoprecipitation assay was applied to verify the existence of the physical association between caspase-8 small subunit and Bcl-2. In CHO cells, the endogenous Bcl-2 was found to co-immunoprecipitate with the ectopically expressed EGFP-fused p10 subunit (Fig 6*B*). These results strongly suggest that Bcl-2 is capable of associating with caspase-8, which may facilitate its cleavage by caspase-8 during OSW-1 induced apoptosis.

In addition, we also determined whether the interaction between the small subunit of caspase-8 and Bcl-2 might have functional consequences *in vivo*. Indeed, Bcl-2 cleavage was partially inhibited in OSW-1 treated CHO cells upon transient overexpression of p10 (Fig. 7*A*), accompanied by the reduction of the number of apoptotic cells (Fig. 7*B*). We surmise that the overexpressed caspase-8 small subunits in the OSW-1 treated cells compete with the endogenous caspase-8 molecules to the binding site of Bcl-2 molecules, thereby preventing active caspase-8 molecules from cleaving Bcl-2 proteins.

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Discussion

Although it has been reported by some groups that the Bcl-2 cleavage was carried out by caspase-3 (Cheng et al., 1997), this notion was challenged by the observation that the cleavage of Bcl-2 was detected in caspase-3 mutant MCF-7 cells (Kim et al., 1998). It has been reported that several purified recombinant human caspases such as caspase-1, -5, -7, -8 can cleave Bcl-2 *in vitro* (Kim et al., 1998). Furthermore, Bcl-2-coated beads could sequester procaspase-8 in cell lysates by the formation of caspase-8/Bcl-2 complex (Poulaki et al., 2001). These results are consistent with our observation, that Bcl-2 cleavage was mediated by caspase-8 rather than by caspase-3 in mammalian cells upon treatment with OSW-1.

An important remaining question is how caspase-8 is activated in the cells treated with OSW-1. It has been known that activation of caspase-8 is mediated through the death receptor pathway (Thorburn, 2004). We addressed this question, at least in part, by treating Jurkat T cells either deficient in FADD or caspase-8 with OSW-1. The significant suppression of the cell death in either FADD or caspase-8 mutant cells suggests that the death receptor pathway is involved in the OSW-1 induced apoptosis (Fig. 5*B*). However, caspase-8 was still slightly activated in FADD-/- cells, while the cell death induced by OSW-1 in FADD-/- cells was more pronounced than that in caspase-8-/- cells (Fig. 5*B*), suggesting that except for the Fas/FADD receptor pathway there is another route activating caspase-8, which might be independent of the death receptor pathway (Ryu et al., 2005).

The interplay between the mitochondrial and the death receptor-mediated pathways is regulated by Bcl-2 family. A BH-3 only protein Bid has been identified to provide the link between these two pathways, which is cleaved by caspase-8 and translocated to mitochondria to play its pro-apoptotic role (Li et al., 1998). The present work suggests that caspase-8 directly

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participates in the cleavage of Bcl-2, which makes a new link for the cross-talk between the mitochondrial and the death receptor-mediated pathways.

Since it was observed that the cleavage of Bcl-2 by caspase-8 took place prior to the Bax translocation in the OSW-1 treated CHO cells (data not shown), we suggest that caspase-8 dependent cleavage of Bcl-2 contributes to the amplification of death signals through promoting the translocation of Bax from cytosol to mitochondria, which results in the release of cytochrome *c* from mitochondria. On the other hand, we can not exclude the possibility that caspase-8 mediated cleavage converts Bcl-2 to a pro-apoptotic fragment as previously described (Cheng et al., 1997), which might directly promote the cytochrome *c* release from mitochondria.

It has been known that both the control of cell proliferation and the regulation of apoptosis are disregulated during cancer development (Evan and Vousden, 2001). A higher incidence of genetic alterations of apoptotic mediators occurs in malignant tumors, such as Bcl-2 overexpression (Raffo et al., 1995) or Apaf-1 inactivation (Soengas et al., 2001). Therefore, a promising strategy for developing new cancer chemotherapy is to develop anticancer drugs that either activate apoptosis or increase the susceptibility to apoptosis among malignant cells (Bamford et al., 2000; Kaufmann and Earnshaw, 2000). Our results demonstrate that OSW-1 belongs to this class of apoptosis-inducing agents, raising the possibility that OSW-1 could be developed as a potential anti-tumor drug.

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Footnotes

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Figure Legends

Figure 1 Apoptosis of CHO cells is induced by OSW-1. **A**, detection of apoptotic CHO cells in the presence of OSW-1. CHO AA8 cells were treated with 200 ng/ml OSW-1 for 24 h. Then the treated cells were subjected to sub-G1 analysis (upper panel in the left), annexin-V analysis (lower panel in the left) and DNA-fragmentation analysis (right panel). * compared with control, $p<0.05$. **B**, OSW-1 induces apoptosis in a dose- and time- dependent manner. Flow cytometric analysis was used to detect sub-G1 DNA content of cells incubated with OSW-1 at indicated concentrations for 24 h (upper panel) or with 100 ng/ml OSW-1 for indicated times (lower panel). ** compared with control $p<0.01$, ANOVA followed by *post hoc* test.

Figure 2 OSW-1 induced apoptosis is involved in mitochondria-mediated pathway. **A**, analysis of Bcl-2 cleavage, Bax translocation and cytochrome *c* release during the apoptotic process in CHO AA8 cells. After the drug treatment, the floating cells taken as apoptotic cells in the late stage of apoptosis were harvested and fractionated as described in Materials and Methods. The total cell lysates (lanes 1 and 4), cytosolic fractions (lanes 2 and 5) and mitochondrion-fractions (lanes 3 and 6) were subjected to Western blotting. □Bcl-2 is a cleaved form of Bcl-2 about 23 Kd. Cytochrome *c* oxidase IV (Cox 4) was shown as a quality control for fractionations. **B,** overexpession of Bcl-2 in the stably transfected CHOC 400 cells. The subcellular localization of expressed EGFP-Bcl-2 fusion protein was analyzed. The total cell lysates (lanes 1 and 4), cytosolic fractions (lanes 2 and 5) and mitochondrion-fractions (lanes 3 and 6) were subjected to Western blotting with an anti-GFP antibody. **C**, cleavage of endogenous Bcl-2 and the translocation of Bax and cytochrome *c* are blocked in the cells overexpressing Bcl-2. After the treatment with 100 ng/ml OSW-1 for 30 h, the transfected cells and the control cells were harvested and fractionated. The total cell lysates (lanes 1, 4, 7 and 10), cytosolic fractions (lanes

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2, 5, 8 and 11) and mitochondrion-fractions (lanes 3, 6, 9 and 12) were subjected to Western blotting. **D**, OSW-1 induced apoptosis is inhibited by overexpressing Bcl-2. The sub-G1 DNA contents of treated cells were analyzed by flow cytometer. ** $p<0.01$, ANOVA followed by *post hoc* test.

Figure 3 OSW-1 induced Bcl-2 cleavage is independent of caspase-3. **A**, Bcl-2 cleavage occurs prior to the activation of caspase-3 in the presence of OSW-1. CHO AA8 cells were treated with 200 ng/ml OSW-1 for indicated times. The cleavage of Bcl-2 and procaspase-3 were analyzed by Western blotting. A p19 fragment was detected as the active form of caspase-3. **B**, OSW-1 induces Bcl-2 cleavage in the presence of a caspase-3 inhibitor. The CHO cells treated with 40 μM caspase-3 inhibitor DQMD-fmk 1 h, followed by addition of 200 ng/ml OSW-1 and incubation for 24 h. The samples were subjected to Western blotting. The bottom panel shows a p20 fragment that is an immature cleavage product of caspase-3 in the presence of the caspase-3 inhibitor. **C**, OSW-1 induces Bcl-2 cleavage in cells expressing a caspase-3 dominant negative (DN) mutant. CHO AA8 cells were transiently transfected with a mutant caspase-3 plasmid as described in Materials and Methods. The transfected cells were treated with 200 ng/ml OSW-1 for 24 h. The cells were harvested and lysed for Western blotting. The right panel shows the transient expression of mutant caspase-3 protein that is fused with EGFP (EGFP-caspase-3 ^{m}) by Western blotting with anti-caspase-3 antibody.

Figure 4 OSW-1 induced Bcl-2 cleavage is caspase-8 dependent. **A**, inhibition of OSW-1 induced Bcl-2 cleavage in the presence of a caspase-8 inhibitor. CHO AA8 cells were treated with 40 μM caspase-8 inhibitor IETD-cho 1 h, followed by addition of 200 ng/ml OSW-1 and incubation for an additional 20 h. The relative caspase-8 activity was measured (left panel), and the Bcl-2 cleavage was analyzed by Western blotting (right panel). * $p<0.05$, ANOVA followed

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by *post hoc* test. **B**, inhibition of OSW-1 induced Bcl-2 cleavage in the cells stably transfected with caspase-8 dominant negative (DN) plasmid. Stably transfected CHO AA8 cells expressing dominant negative caspase-8 mutant were isolated as described in Materials and Methods. The transfected cells were treated with 200 ng/ml OSW-1 for 20 h. The cleavage of Bcl-2 was analyzed by Western blotting (right panel). The left panel shows the expression of mutant caspase-8 protein fused with FLAG (FLAG-caspase-8^{m}), which was detected by anti-FLAG antibody. **C**, Inhibition of caspase-8 activation reduces the OSW-1 induced apoptosis. The sub-G1 DNA content of cells either in the presence of IETD-cho (left panel) or transfected with caspase-8 DN (right panel) was analyzed with flow cytometer. * $p<0.05$ and ** $p<0.01$, ANOVA followed by *post hoc* test.

Figure 5 Caspase-8 deficient cells are resistant to OSW-1 induced Bcl-2 cleavage and apoptosis. **A**, Bcl-2 cleavage is inhibited in the FADD or caspase-8 mutant cells in the presence of OSW-1. Jurkat T cell lines deficient in FADD or caspase-8 and the wild-type cell line A3 were treated with 100 ng/ml OSW-1 for 12 hr, and analyzed by Western blotting. The upper panel shows a p43/41 fragment that is an active product cleaved from procaspase-8. **B**, Caspase-8 deficient cells are insensitive to apoptosis induced by OSW-1. Sub-G1 DNA contents were detected by flow cytometric analysis after the drug treatment. * $p<0.05$ and ** $p<0.01$, ANOVA followed by *post hoc* test.

Figure 6 Physical Interaction between caspase-8 and Bcl-2. **A**, yeast L40 cells were co-transformed with plasmids for LexA DNA binding domain and Gal4 activation domain fusion proteins as indicated. The bait plasmids containing sequences for both large (p18) and small (p10) subunits of caspase-8 were expressed separately under ADH1 promoters, while the small subunit was fused to LexA DNA binding domain. $p18^{m}$ represents a p18 mutant fragment, which is the

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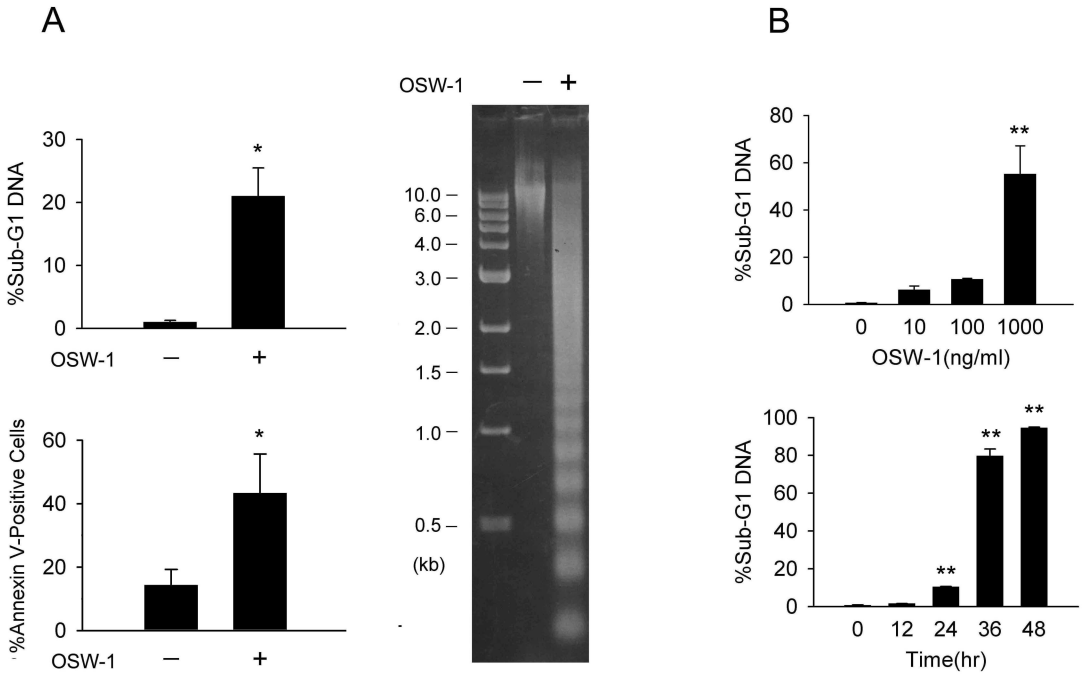
same as caspase-8 DN at the active site in p18 subunit. The expression of reporter gene *Lac Z*, which is induced by the interaction between caspase-8 and Bcl-2 in this yeast two-hybrid system, caused pink yeast clones to change into blue color in the presence of X-gal. "+" and "++" indicate the development of blue color within 2.5 h and 2 h, while "–" indicates no such color development. **B**, co-immunoprecipitation of Bcl-2 and EGFP-p10. The whole cell lysates of CHO cells stably transfected with pEGFP-casp8p10 were immunoprecipitated with anti-Bcl-2 antibody. Immunoprecipitates were immunoblotted by anti-GFP antibody (upper panel), while the amount of immunoprecipitates was controlled with antibody against Bcl-2 (lower panel). IP, immunoprecipitation; WB, Western blotting. **C**, The detection of EGFP-p10 fusion protein. The whole cell lysates of CHO cells stably transfected with pEGFP or pEGFP-casp8p10 were subjected to Western blotting with anti-GFP antibody.

Figure 7 Functional consequence of the interaction between caspase-8 small subunit and Bcl-2 *in vivo*. CHO AA8 cells were transiently transfected with a plasmid expressing EGFP-p10 (caspase-8) or the vector. **A**, Bcl-2 cleavage is partially inhibited by p10 overexpression. The transfected cells were treated with 200 ng/ml OSW-1 for 20 h and analyzed by Western blotting (upper panel). The signals from three independent blots were quantified and analyzed statistically (lower panel). **B**, overexpression of p10 reduces OSW-1 induced apoptosis. The percentage of sub-G1 DNA content was analyzed with flow cytometer. * $p<0.05$, ANOVA followed by *post hoc* test.

Fig.1


A
%Sub-G1 DNA
30
20
10
0
*
OSW-1
–
+
%Annexin V-Positive Cells
60
40
20
0
*
OSW-1
–
+
OSW-1
–
+
10.0
6.0
4.0
3.0
2.0
1.5
1.0
0.5
(kb)
B
%Sub-G1 DNA
80
60
40
20
0
**
0
10
100
1000
OSW-1(ng/ml)
%Sub-G1 DNA
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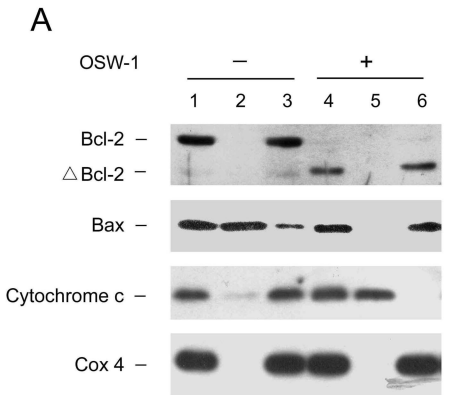
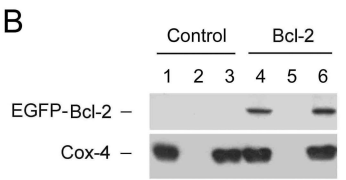
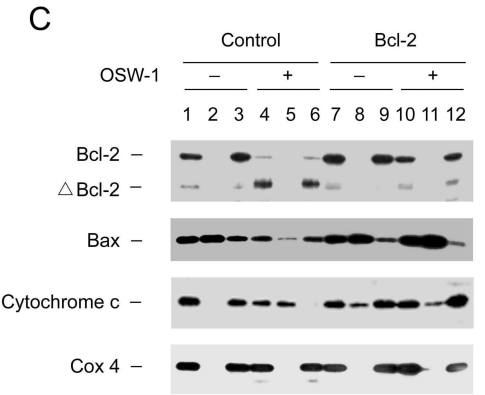
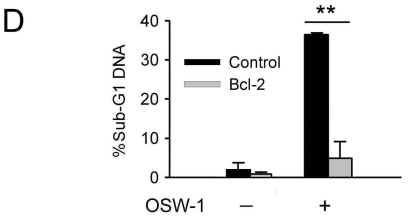
80
60
40
20
0
**
**
**
0
12
24
36
48
Time(hr)

Fig. 2


A
OSW-1
–
+
1 2 3 4 5 6
Bcl-2 –
△Bcl-2 –
Bax –
Cytochrome c –
Cox 4 –


B
Control
Bcl-2
1 2 3 4 5 6
EGFP-Bcl-2 –
Cox-4 –


C
Control
Bcl-2
OSW-1
– + – +
1 2 3 4 5 6 7 8 9 10 11 12
Bcl-2 –
△Bcl-2 –
Bax –
Cytochrome c –
Cox 4 –


D
**
40
30
20
10
0
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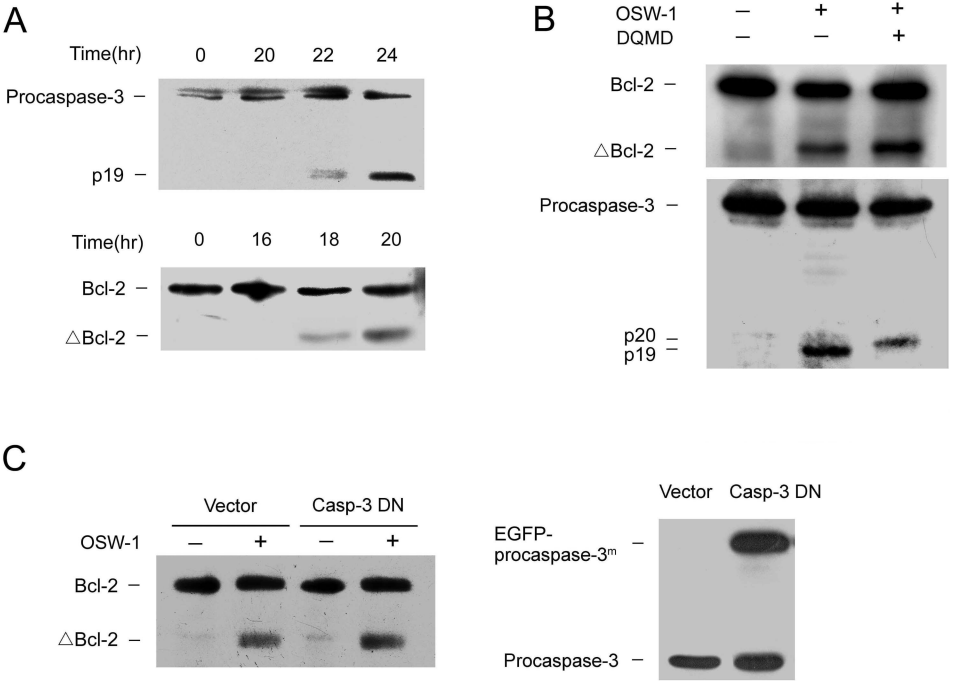
Control
Bcl-2
OSW-1 – +

Fig.3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Time(hr) 0 20 22 24
Procaspase-3
p19
Time(hr) 0 16 18 20
Bcl-2
△Bcl-2
B
OSW-1 – + +
DQMD – – +
Bcl-2
△Bcl-2
Procaspase-3
p20
p19
C
Vector
Casp-3 DN
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Bcl-2
△Bcl-2
Vector Casp-3 DN
EGFP-procaspase-3m
Procaspase-3

Fig.4

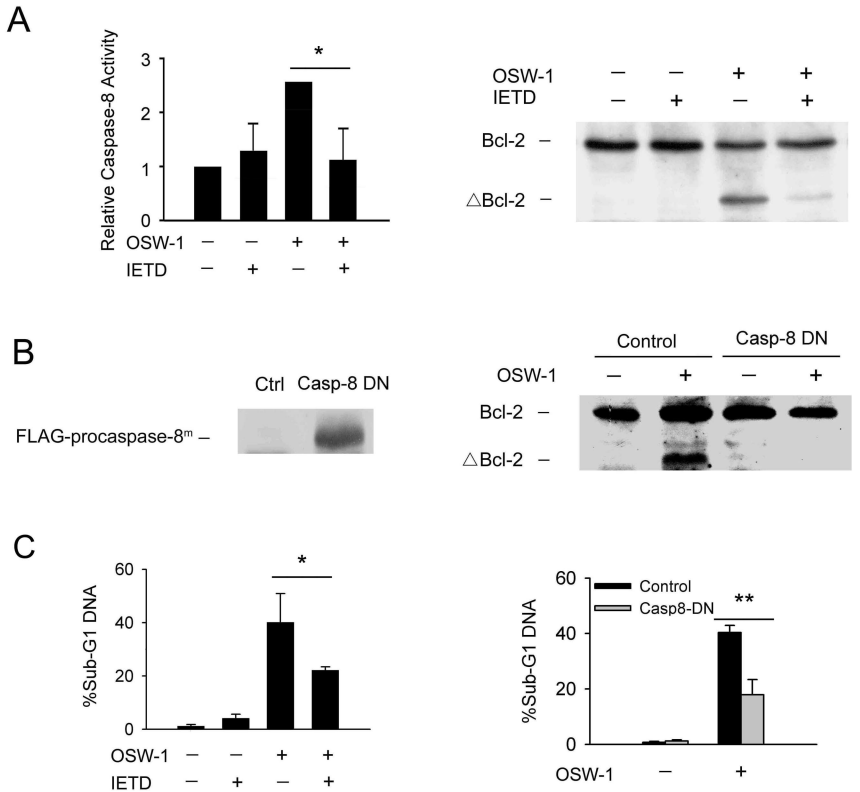
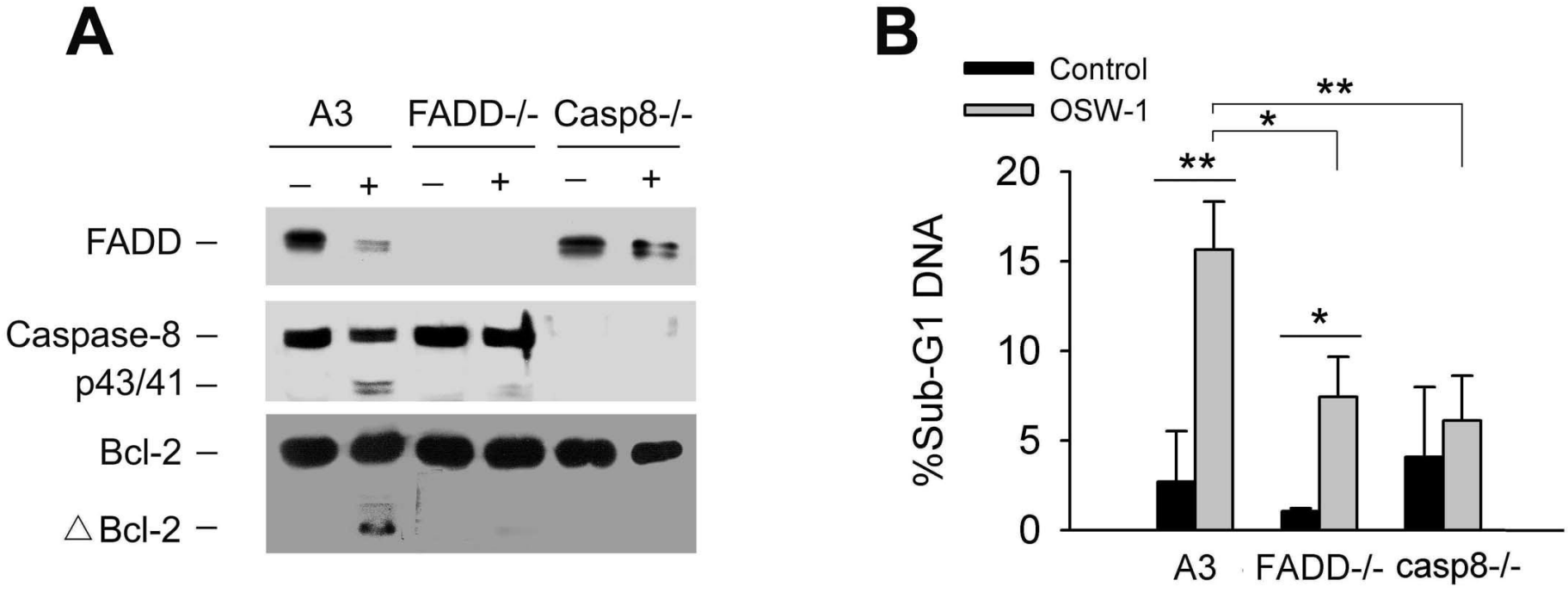

A
Relative Caspase-8 Activity
3
2
1
0
*
OSW-1 – – + +
IETD – + – +
OSW-1 – – + +
IETD – + – +
Bcl-2 –
△Bcl-2 –
B
Ctrl Casp-8 DN
FLAG-procaspase-8m –
Control
Casp-8 DN
OSW-1 – + – +
Bcl-2 –
△Bcl-2 –
C
%Sub-G1 DNA
60
40
20
0
*
OSW-1 – – + +
IETD – + – +
%Sub-G1 DNA
60
40
20
0
Control
Casp8-DN
**
OSW-1 – +

Fig.5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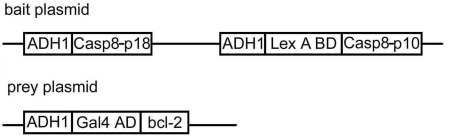
A3
FADD-/-
Casp8-/-
–
+
–
+
–
+
FADD –
Caspase-8 –
p43/41 –
Bcl-2 –
△Bcl-2 –
B
Control
OSW-1
**
*
**
*
20
15
10
5
0
%Sub-G1 DNA
A3
FADD-/-
casp8-/-

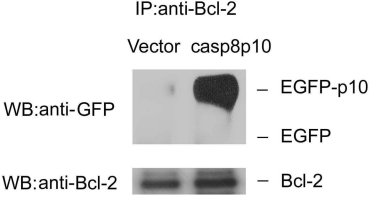
Fig.6

A



pBTM116(LexA DB)-casp8-	pGAD10(Gal4 AD)-	β-Galactosidase activity
p10p18	–	–
p10p18	bcl-2	+
p10p18^{m}	bcl-2	++
p10	–	–
p10	bcl-2	+
p18	–	–
p18	bcl-2	–
p18^{m}	bcl-2	–

B



C

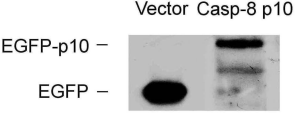
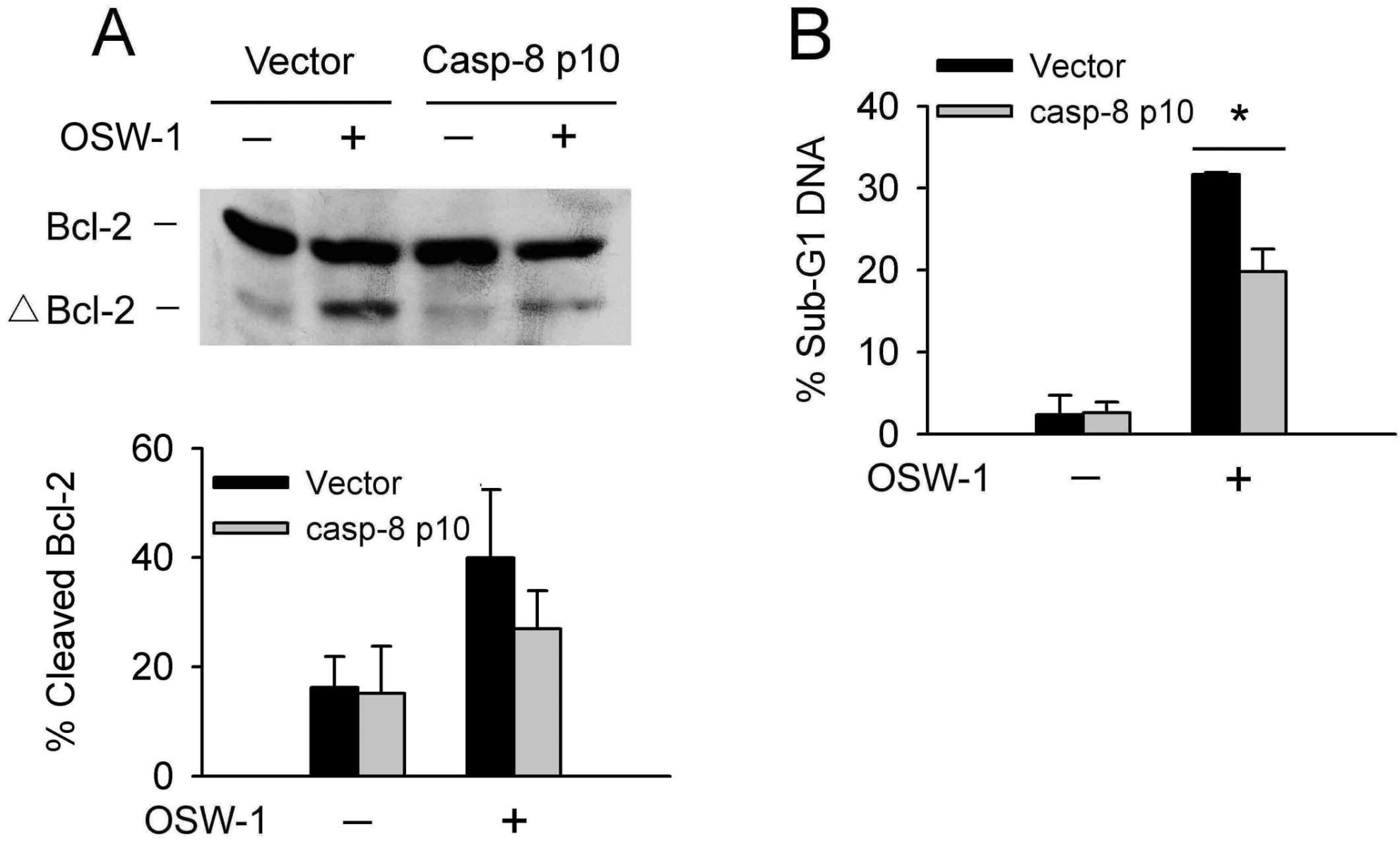


Fig.7


A
Vector
Casp-8 p10
OSW-1
—
+
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+
Bcl-2
△Bcl-2
% Cleaved Bcl-2
60
40
20
0
Vector
casp-8 p10
OSW-1
—
+
B
Vector
casp-8 p10
*
% Sub-G1 DNA
40
30
20
10
0
OSW-1
—
+