Cell Research (2007) 17:441-448.

www.nature.com/cr

ORIGINAL ARTICLE

# Bcl-2 cleavages at two adjacent sites by different caspases promote cisplatin-induced apoptosis

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The protein encoded by *bcl-2* proto-oncogene plays an important role in the mitochondria-mediated apoptotic pathway. Although the general role of Bcl-2 is anti-apoptotic, previous work showed that Bcl-2 fragments cleaved by caspases could promote apoptotic process. We report herein that Bcl-2 protein was cleaved to produce two fragments of around 23 kDa in human hepatocarcinoma BEL-7404 cells or in Bcl-2 overexpressing CHO cells induced by cisplatin. Treating cells with the general caspase inhibitor z-VAD-fmk blocked the induced cleavage of Bcl-2. Mutagenesis analyses showed that Bcl-2 was cleaved by caspases at two adjacent recognition sites in the loop domain ($YEWD^{31}\downarrow AGD^{34}\downarrow V$), which could be inhibited by caspase-8 and -3 inhibitors, respectively. Overexpression of the carboxyl terminal 23 kDa fragments increased the sensitivity of CHO cells to cisplatin-induced apoptosis. These results indicate that Bcl-2 can be cleaved into two close fragments by different caspases during cisplatin-induced apoptosis, both of which contribute to the acceleration of apoptotic process.

***Keywords*:** Bcl-2, apoptosis, cisplatin, caspase-3, caspase-8

*Cell Research* (2007) **17**:441-448. doi: 10.1038/cr.2007.36; published online 24 April 2007

## Introduction

Apoptosis is a strictly controlled cellular process and plays an important role in the normal development of multicellular organisms as well as in pathological processes [1, 2]. There are two main pathways leading to apoptotic cell death. The extrinsic apoptotic pathway is activated by the interaction of death receptors on cell surface with their ligands, while the second pathway involves the participation of mitochondria and is regulated by members of the Bcl-2 family [3]. Anti-apoptotic members of Bcl-2 family such as Bcl-2 and Bcl-xL inhibit the release of cytochrome c from mitochondria, while pro-apoptotic members such as Bax and Bak decrease the mitochondrial membrane potential ($\Delta\psi$) and accelerate cytochrome c release to trigger downstream apoptotic events [4, 5].

The aspartate specific cysteine proteases called caspases amplify apoptosis by an activation cascade [6]. The activated caspases in turn cleave various cellular substrates, resulting in the characteristic biochemical and morphological changes associated with apoptosis [7]. For example, active caspase-8 could cleave a pro-apoptotic Bcl-2 family member Bid to generate tBid, which then translocates to mitochondria to facilitate cytochrome c release [8]. When caspase-3 is activated, it cleaves several important proteins related to apoptosis such as the DNA repair enzyme poly-(ADP-ribose) polymerase (PARP) [9] and DNA fragmentation factor 45 (DFF45) [10]. An increasing number of caspase substrates have been identified.

It has been reported that Bcl-2 is cleaved in the loop domain by caspase during apoptosis, which results in the inactivation of Bcl-2 [11] or even converts Bcl-2 to a Bax-like fragment [12]. Structural and functional analysis of the cleaved fragment revealed that the $NH_2$-terminal

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Received 19 November 2006; revised 19 January 2007; accepted 11 February 2007; published online 24 April 2007

region of Bcl-2 is required for its anti-apoptotic activity and heterodimerization with the pro-apoptotic protein Bax [13]. Inhibition of caspase-mediated cleavage of Bcl-2 results in suppression of apoptosis [14]. Some studies demonstrated that caspase-3 was responsible for the Bcl-2 cleavage [11, 12 and 15]. However, Bcl-2 cleavage has been shown to occur in caspase-3 deficient MCF-7 cells [14]. Recently, our laboratory reported a caspase-8-dependent cleavage of Bcl-2 in Chinese hamster ovary (CHO) cells [16].

In the course of examining cisplatin-induced apoptosis, we found that Bcl-2 was cleaved by different caspases at two adjacent recognition sites of $Asp^{31}$ and $Asp^{34}$ in the loop domain. Furthermore, both forms of the truncated Bcl-2 fragments increased the sensitivity of transfected cells to apoptotic stimulus.

## Materials and Methods

*Cell culture*

CHO AA8 cells and human breast adenocarcinoma MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum and 100 μM nonessential amino acids (Gibco BRL). Stable subclone cells of CHO AA8 that overexpress dominant-negative caspase-8 (16) were maintained in complete medium containing 500 μg/ml G418. Human hepatocarcinoma BEL-7404 cells were grown in DMEM medium supplemented with 10% fetal bovine serum.

*Plasmid construction*

The pEGFP vector containing wild-type human *bcl-2* was described previous [16]. Substitutions of aspartic acids at 31 and 34 with alanine (D31A, D34A and D31, 34A) were accomplished by converting the aspartic acid codon GAT to alanine codon GCT by PCR mutagenesis. The following mutagenesis primers were used to generate *bcl-2* site-directed mutants: 5'-TAC GAG TGG GA(C)T GCG GGA GA(C)T GTG GGC GCC-3' and 5'-GCC CCT CTG CGA CAG CTT ATA-3'. The $NH_2$ terminal-truncated form of human Bcl-2 (ΔN31, lacking $NH_2$-terminal 1-31 amino acids; ΔN31D34A, ΔN31 with a D34A mutation; and ΔN34, lacking $NH_2$-terminal 1-34 amino acids) were generated from pEGFP-*bcl-2* by PCR and cloned into pcDNA4/TO vector (Invitrogen). The caspase-3 construct was generated by inserting human caspase-3 cDNA into a pcDNA3.1 vector.

*Transient transfection*

CHO cells were transiently transfected with constructs expressing either the truncated Bcl-2 or full length human Bcl-2 fused to EGFP by electroporation with Nucleofector T kit according to the manufacturer's instructions (Amaxa Biosystems). Transfection of caspase-3 expression vector into MCF-7 cells was conducted with lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. The transfection efficiencies were up to 70%.

*Preparation of cytosolic and mitochondrial extracts by digitonin treatment*

CHO cells were harvested and resuspended in a buffer (20 mM Hepes-KOH, pH 7.3, 110 mM KAc, 5 mM NaAc, 2 mM $MgAc_2$, and 1 mM EGTA) containing 200 μg/ml digitonin (Calbiochem) on ice for 10 min. The permeabilized cells containing cellular organelles and nuclei were pelleted as mitochondria-containing fractions, and the supernatants were collected as cytosolic fractions.

*Western-blotting analysis*

Intact cells, the supernatants, and the mitochondria-containing fractions were lysed in loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol, and 0.1% bromphenol blue). Equalized amounts of proteins from each sample were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. Western-blotting analysis was carried out by incubation of the membrane with one of the following primary antibodies: anti-cytochrome c, anti-GFP, anti-Actin and anti-caspase-8 (Santa Cruz Biotechnology); anti-caspase-3 (Cell Signaling); anti-Bcl-2 (Sigma-Aldrich), and followed by incubation with horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology). Immune complexes were detected by the enhanced chemiluminescence system according to the manufacturer's instructions (ECL; GE Healthcare).

*Flow cytometric analysis*

To identify sub-G1 DNA region (below the G0/G1 peak) that is indicative of cells undergoing apoptosis, the drug-treated cells were harvested and fixed with 70% ethanol. The fixed cells were digested with RNase A, stained with propidium iodide and analyzed by flow cytometer (FACScan; BD Biosciences).

*Statistical analysis*

All the data in this study were expressed as mean ± standard error (SE) from at least three independent experiments and the comparisons of means were performed using the paired *t*-test. A value of $p<0.05$ was considered statistically significant.

## Results

*Cleavage of Bcl-2 protein during cisplatin-induced apoptosis*

Cisplatin is a potent inducer of apoptosis and widely used as a chemotherapeutic agent [17]. It was used here to induce apoptosis in human hepatocarcinoma BEL-7404 cells (Figure 1A). We examined the changes of Bcl-2 protein in cisplatin-treated cells by Western-blotting analysis. Two cleaved bands of about 23 kDa were clearly detected by the anti-human Bcl-2 monoclonal antibody when the cells were treated with 10 μg/ml or higher concentrations of cisplatin (Figure 1B). Moreover, the drug-induced cleavage of Bcl-2 could be inhibited by preincubation with a pan-caspase inhibitor z-VAD-fmk (Figure 1B, last lane).

To confirm that the Bcl-2 protein was cleaved to produce two fragments of about 23 kDa in the presence of cisplatin, we transfected CHO cells with EGFP-fused human Bcl-2 (hBcl-2). The expression of exogenous hBcl-2 was confirmed by Western-blotting with anti-hBcl-2 and anti-GFP, respectively (Figure 2A). There was no interference of endogenous Bcl-2 of CHO cells in Western-blotting analysis because of no cross-reaction between the anti-hBcl-2

mAb and the hamster Bcl-2 protein. After treatment with cisplatin for 24 h, two cleaved fragments of EGFP-fused human Bcl-2 protein were detected (Figure 2A, upper panel). When the drug treatment was prolonged to 48 h, the intensity of cleaved fragments increased (Figure 2A, upper panel). Both cleaved products could be eliminated by preincubation with the inhibitor z-VAD-fmk (Figure 2A, upper panel), indicating that these two small fragments of Bcl-2 were likely generated via cleavage by caspases. Although the level of total Bcl-2 was higher in the cisplatin-treated cells compared to the untreated cells (Figure 2A, upper panel, compare lane 3, 4, 6, 7 with lane 2), there was no significant difference in Bcl-2 abundance between the 24 h-drug-treated cells and 48h-drug-treated cells, suggesting that the increment of Bcl-2 cleavage at 48 h is determined by the increased activity of caspases, but not

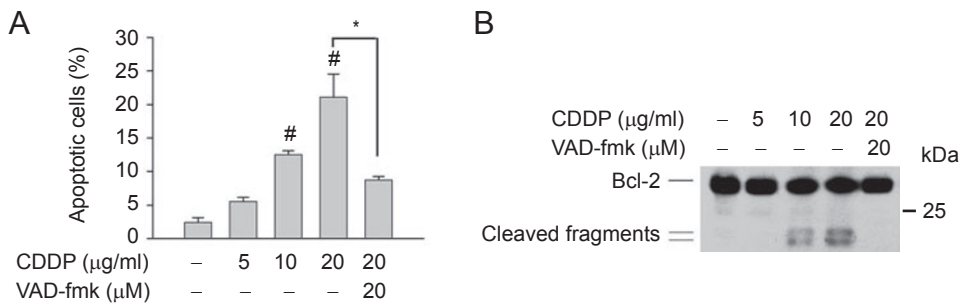


**Figure 1** Bcl-2 cleavage in BEL-7404 cells. **(A)** Apoptosis induced by cisplatin (CDDP) in BEL-7404 cells. After treatment with cisplatin at different concentrations for 24 h, the percentage of cells with a sub-G1 DNA content (taken as apoptotic cells) were measured by flow cytometry. The apoptotic cells were reduced by 1 h of preincubation with a pan-caspase inhibitor z-VAD-fmk (Promega). $^{\#}p<0.05$, compared with control; $^{*}p<0.05$, paired t-test. **(B)** Two fragments of Bcl-2 cleavage were detected in cisplatin-treated BEL-7404 cells, and such cleavage was inhibited by z-VAD-fmk. The samples were separated by SDS-PAGE on an 12% acrylamide gel and detected with anti-hBcl-2 mAb.

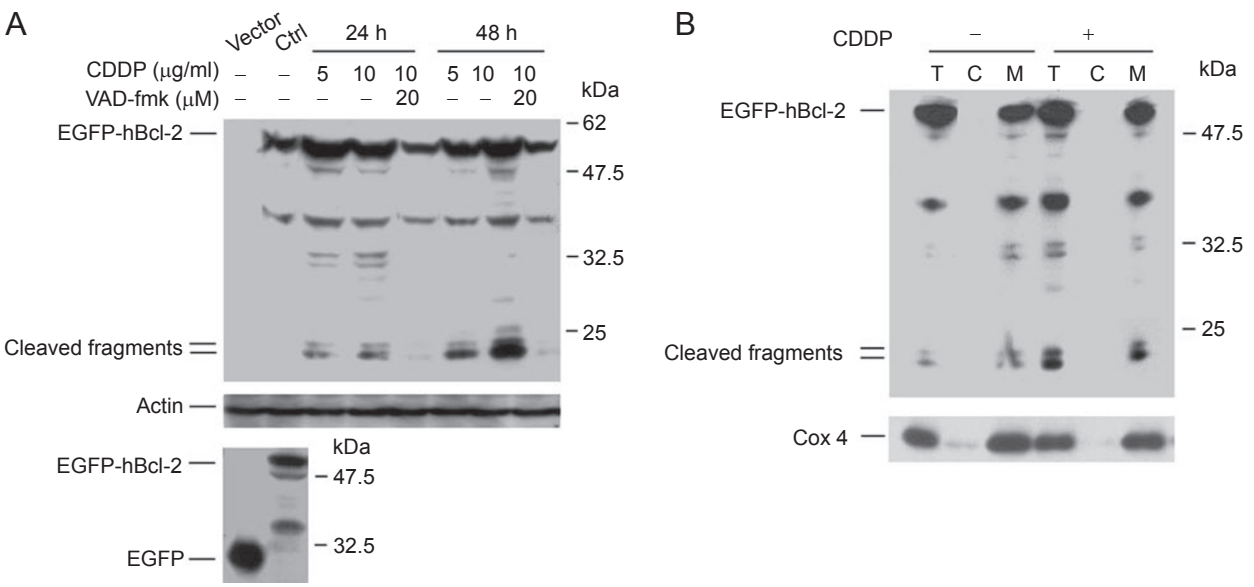


**Figure 2** Bcl-2 cleavage in hBcl-2-overexpressing CHO cells. **(A)** The overexpression of EGFP-fused hBcl-2 protein was confirmed by Western-blotting analysis using anti-hBcl-2 and anti-GFP antibodies, respectively. Two fragments of Bcl-2 cleavage were detected in hBcl-2 transfected CHO cells in the presence of cisplatin (CDDP), and such cleavage was inhibited by preincubation with z-VAD-fmk. Actin was used as sample-loading control. **(B)** The subcellular location of cleaved fragments. After drug treatment, cells were fractionated as described in Materials and Methods. Total cell lysates (T), cytosolic fractions (C) and mitochondrion-containing fractions (M) were subjected to Western-blotting. Cytochrome c oxidase IV (Cox 4) was shown as a quality control for fractionations.

by the expression level of excessive Bcl-2.

Bcl-2 possesses a C-terminal transmembrane domain that localizes the protein to mitochondrial membrane [18]. If the cleavage is executed at C-terminus, the truncated Bcl-2 protein must be released from the membrane. To analyze the location of the truncated products in cells, we fractionated Bcl-2 over-expressing CHO cells to obtain cytosolic and mitochondrion-containing fractions by the digitonin-permeabilization method (see Materials and Methods). The results showed that drug-induced cleaved fragments of Bcl-2 remained in the mitochondrion-fractions (Figure 2B), suggesting that they retained the C-terminus of Bcl-2 but lost the N-terminus.

*Determination of the cleavage sites in Bcl-2 protein*

There are several aspartic acid residues in the N-terminus of hBcl-2 protein, ($Asp^{10}$, $Asp^{31}$, $Asp^{34}$ and $Asp^{64}$) that are candidate cleavage sites for caspases (Figure 3A). However, only the cleavages at $Asp^{31}$ and $Asp^{34}$ would make Bcl-2 protein lose its BH4 domain (Figure 3A) and yield two close fragments of about 23 kDa. It has been reported that $Asp^{34}$ is the substrate site of caspase-3 [12]. We then tried to identify the actual cleavage sites in hBcl-2 by constructing several mutants in which $Asp^{31}$ (D31A) and $Asp^{34}$ (D34A) were substituted by alanine, respectively or simultaneously (D31,34A). Constructs expressing the wild-type and mutant forms were transfected into CHO cells. After cisplatin treatment, the D34A mutant protein was cleaved to yield the larger fragment, whereas the D31A mutant protein was slightly cleaved to yield the smaller fragment (Figure 3B). However, cleavages were not detected in the D31,34A double mutant (Figure 3B). These results indicated that Bcl-2 protein was cleaved at both sites of $Asp^{31}$ and $Asp^{34}$ ($YEWD^{31}\downarrow AGD^{34}\downarrow V$) as expected.

Then CHO cells overexpressing wild-type or mutant Bcl-2 molecules were analyzed for the effects of these mutations on apoptosis under cisplatin treatment. Both D31A and D34A exhibited enhanced protective effects on apoptosis compared with wild-type Bcl-2 (Figure 3C). The decrease of apoptosis was more obvious in cells expressing the D31,34A double mutant in which the cleavage was completely abolished (Figure 3C). These results suggest that cleavages of Bcl-2 promote cisplatin-induced apoptotic cell death.

*Involvement of caspase-8 and caspase-3 in the cisplatin-induced Bcl-2 cleavage*

Since the $DAGD^{34}$ sequence fits with the caspase-3-

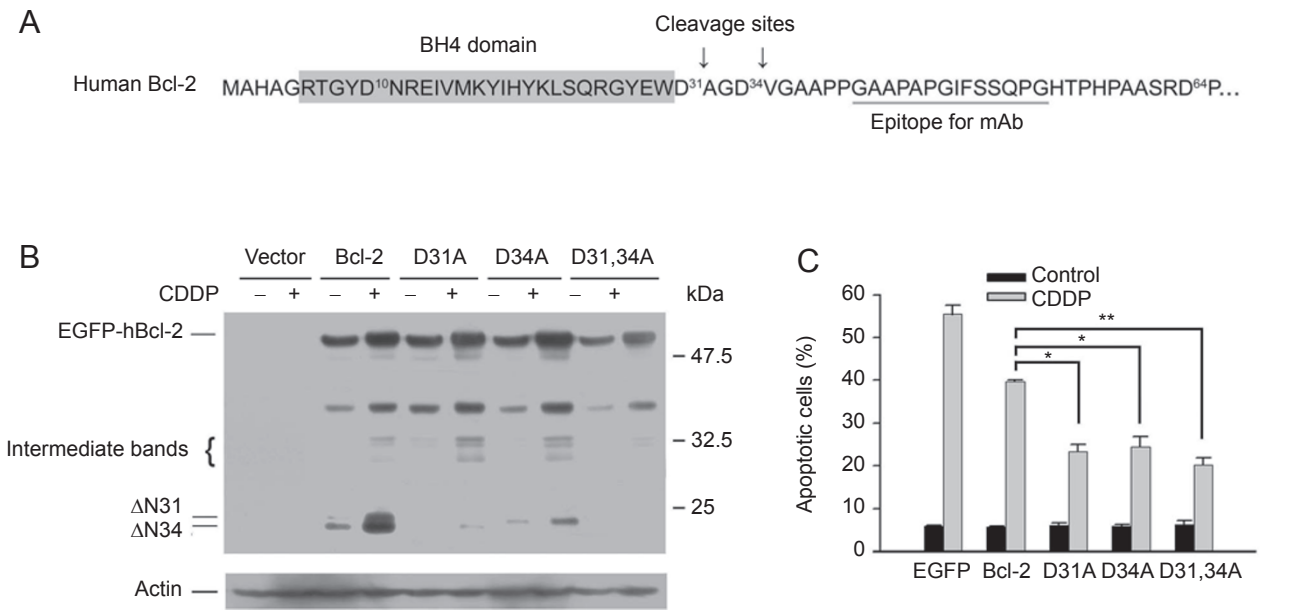


**Figure 3** Identification of the cleavage sites. **(A)** Amino acid sequence of $NH_2$-terminus of human Bcl-2 protein containing two putative caspase cleavage sites: $Asp^{31}$ and $Asp^{34}$. **(B)** The effect of site-directed mutations of $Asp^{31}$ and $Asp^{34}$ on Bcl-2 cleavage. CHO cells overexpressing wild-type Bcl-2 or mutant Bcl-2s (D31A, D34A and D31, 34A) were treated with 10 μg/ml cisplatin (CDDP) for 24 h and then analyzed by Western-blotting assay. Actin was used as the sample-loading control. **(C)** The effect of cleavage-defective mutant Bcl-2s on cisplatin-induced apoptosis. Cells with sub-G1 DNA contents after 48 h CDDP treatment were measured by flow cytometry. $^{*}p<0.05$ and $^{**}p<0.01$, paired t-test.

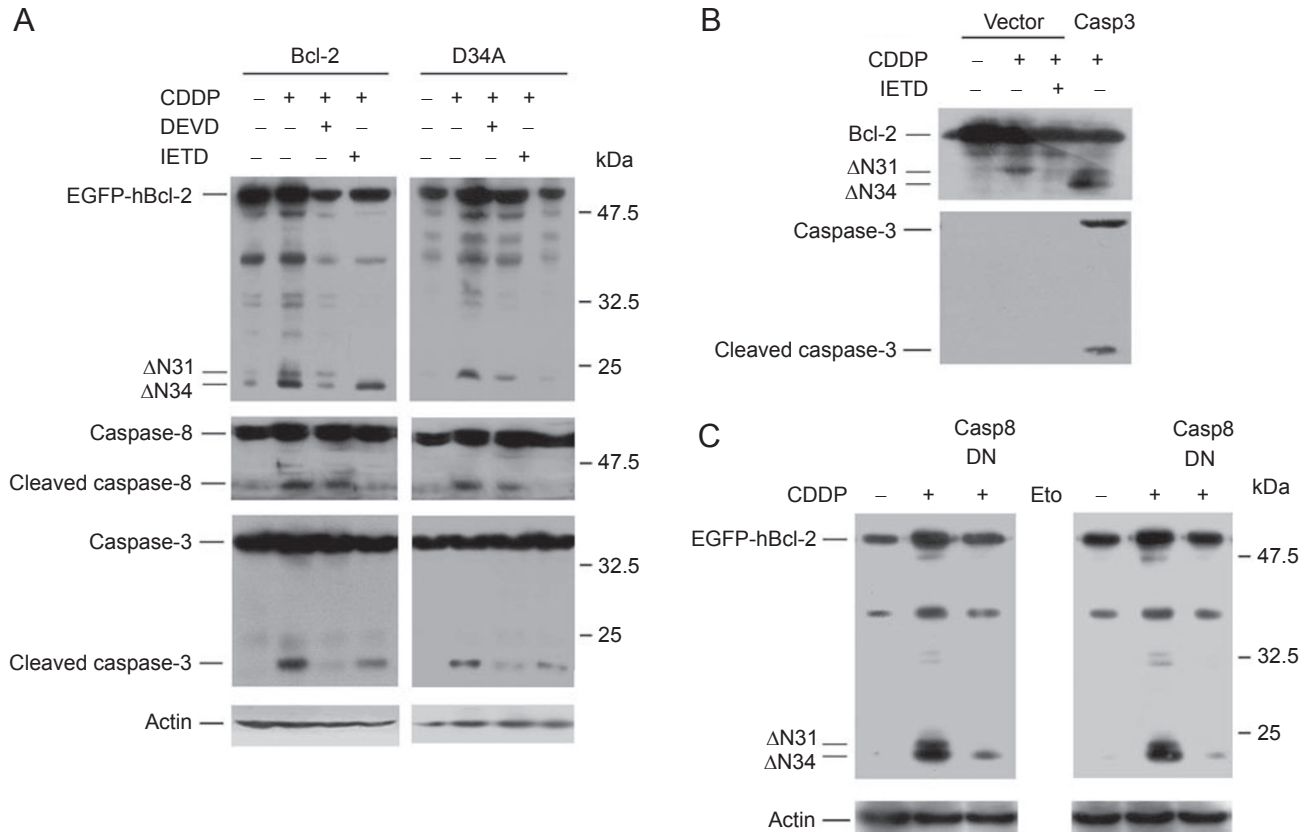


**Figure 4** Involvement of caspase-8 and caspase-3 in Bcl-2 cleavage. **(A)** The two cleavages of Bcl-2 were inhibited by the caspase-8 inhibitor CP-IETD-cho (Calbiochem) and caspase-3 inhibitor CP-DEVD-cho (Calbiochem), respectively. CHO cells expressing wild-type Bcl-2 or D34A mutant were preincubated with 20 μM inhibitors for 1 h and treated with 10 μg/ml cisplatin (CDDP) for 24 h. The activities of caspase-8 and caspase-3 in the absence/presence of the inhibitors were examined by Western-blotting analysis. Actin was used as the sample-loading control. **(B)** The cleavage in caspase-3 reconstituted MCF-7 cells. MCF-7 cells transfected with the caspase-3 expression construct or control vector were treated with 10 μg/ml CDDP for 36 h, or the caspase-3-deficient MCF-7 cells were preincubated with 20 μM IETD-cho before the CDDP-treatment. **(C)** The cleavage in dominant-negative caspase-8 (Casp8 DN) overexpressing cells. The stable transfectants and control cells were transiently transfected with wild-type Bcl-2, then treated with 10 μg/ml CDDP for 24 h (left panel) or 100 μM etoposide (Eto) for 48 h (right panel), respectively. Actin was used as the sample-loading control.

like protease recognition motif DXXD and mutagenesis analysis suggested that $Asp^{31}$ is required for cleavage at $Asp^{34}$ (Figure 3B), we hypothesized that cleavage at $Asp^{34}$ is executed by a caspase-3-like protease. The sequence of $YEWD^{31}$ is not an optimal target site for caspase-3 [19], and it is likely to be cleaved by an unidentified caspase. The large fragment (named ΔN31) was produced in early period (24 h), while an increasing amount of the small fragment (named ΔN34) was detected upon longer drug-treatment (48 h) (Figure 2A), suggesting that cleavage at $Asp^{31}$ was carried out by a caspase preceding caspase-3 activation. Our previous study reported caspase-8 mediated cleavage of Bcl-2, which was independent of caspase-3 [16]. To explore if caspase-8 and caspase-3 were involved in cisplatin-induced Bcl-2 cleavage, we used the caspase-8 specific inhibitor IETD-cho and the caspase-3 specific inhibitor DEVD-cho to suppress the corresponding proteases activities [20, 21]. As shown in Figure 4A, ΔN34 was reduced to background level in the presence of DEVD-cho (lane 3), and ΔN31 was eliminated when cells overexpressing either wild-type Bcl-2 (lane 4) or mutant D34A (lane 8, in which ΔN34 is absent) were pre-incubated with IETD-cho.

MCF-7 is a cell line deficient of caspase-3 [22], which was used to further investigate the mechanism of Bcl-2 cleavage. We reconstituted caspase-3 in MCF-7 cells to restore its function. When cells transfected with vector control were treated with cisplatin, only one cleaved band (should be ΔN31) was detected, and its production could be

inhibited by IETD-cho. In caspase-3 reconstituted MCF-7 cells, besides the former cleaved band, a smaller cleaved band (should be ΔN34) was produced, consistent with the notion that it was generated by caspase-3 (Figure 4B).

To further confirm the role of caspase-8 in Bcl-2 cleavage, a CHO cell line stably expressing dominant-negative (DN) caspas-8 (16) was used, in which caspase-8 activity was specifically inhibited. After cisplatin treatment, the cleaved product ΔN31 was not detected, while ΔN34 was partially reduced (Figure 4C, left panel). To find out if such caspase-8-mediated cleavage was specific to cisplatin induced apoptosis, we further used another chemotherapeutic reagent etoposide as the apoptosis inducer, which activates the caspase-8-mediated apoptotic pathway [23, 24]. Similar results were obtained (Figure 4C, right panel), suggesting that this kind of caspase-8-mediated cleavage of Bcl-2 occurs generally once caspase-8 is activated by apoptotic stresses.

Taken together, these results argue that caspase-8 is involved in the cleavage at $Asp^{31}$, while cleavage at $Asp^{34}$ might be mediated by caspase-3.

*Acceleration of apoptosis by the cleaved Bcl-2 fragments*

Previous work reported that the single cleaved fragment of Bcl-2 promoted apoptosis [12, 16]. To explore the function of these two cleaved fragments of Bcl-2, we constructed vectors individually expressing each of the cleaved Bcl-2 fragments: ΔN31 (losing amino acids 1-31), ΔN31D34A (ΔN31 with a D34A mutation), and ΔN34 (losing amino acids 1-34). These constructs were transiently transfected into CHO cells. Western-blotting analysis showed that the positions of truncated proteins on gel were consistent with the size of the products cleaved from EGFP-Bcl-2 (Figure 5A). In response to cisplatin stimulation, the expression of truncated Bcl-2 proteins were upregulated but no further cleavage at $Asp^{34}$ was detected in ΔN31 (Figure 5A). Fur-

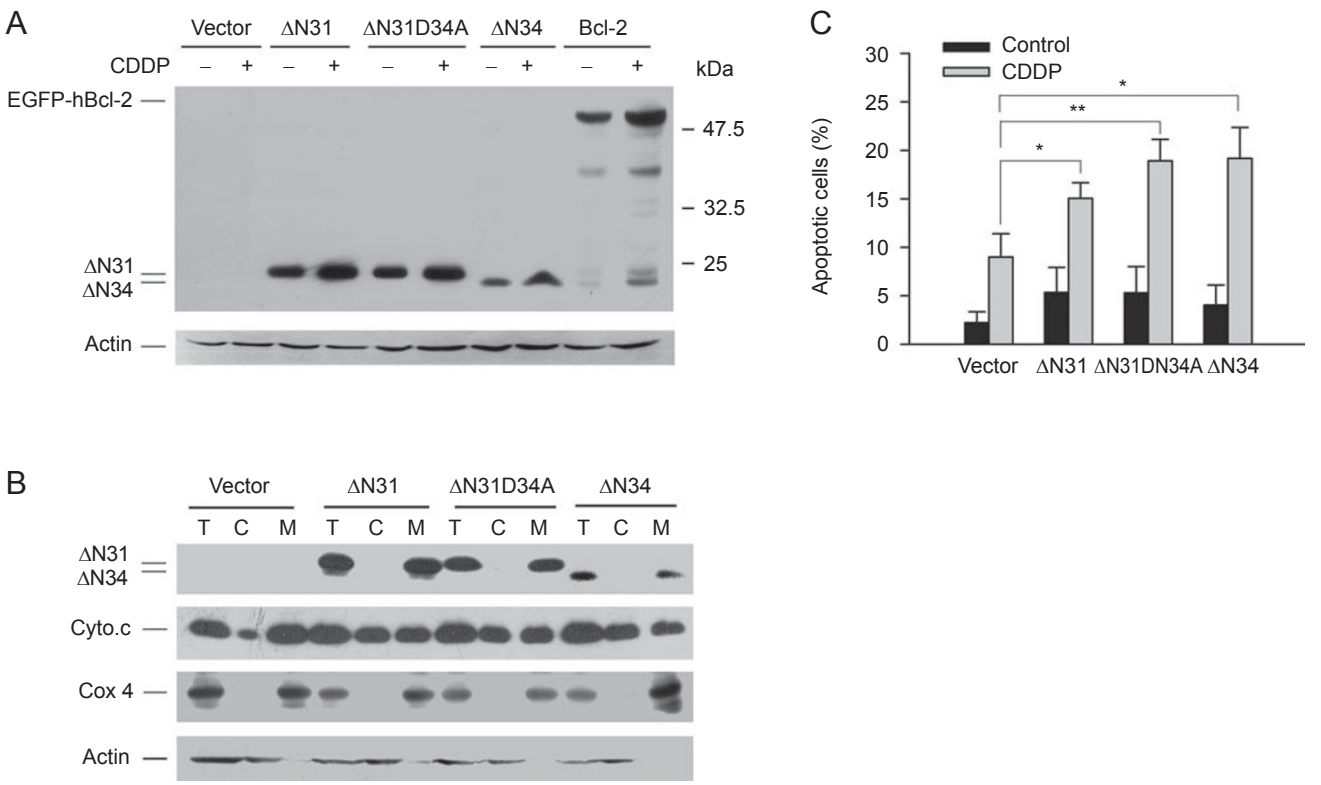


**Figure 5** The truncated Bcl-2 proteins promoted cytochrome c release and accelerated apoptosis. **(A)** Expression of truncated Bcl-2 proteins including ΔN31 (amino acids 32-239), ΔN31D34A (ΔN31 with a D34A mutation) and ΔN34 (amino acids 35-239). Actin was used as the sample-loading control. **(B)** The truncated Bcl-2 fragments promoted cytochrome c release. After treatment with 10 μg/ml cisplatin (CDDP) for 12 h, the apoptotic cells were harvested and fractionated. Total cell lysates (T), cytosolic fractions (C) and mitochondrion-containing fractions (M) were subjected to Western-blotting. Cytochrome c oxidase IV (Cox 4) and actin were shown as mitochondrial and cytosolic markers to control the quality of fractionation. **(C)** The truncated Bcl-2 proteins accelerated cell death. Cells with sub-G1 DNA contents were analyzed by flow cytometry. $^{*}$ $p<0.05$ and $^{**}$ $p<0.01$, paired t-test.

thermore, cell fractionation assay showed that the expressed truncated proteins were all detected in the mitochondrion-fractions (Figure 5B), suggesting that they could function like naturally cleaved Bcl-2 fragments.

We then examined the apoptotic status of cisplatin-treated CHO cells overexpressing these truncated Bcl-2 proteins. The percentage of apoptotic cells was significantly increased by transfection with any of the N-terminal truncated Bcl-2, comparing to the vector control (Figure 5C). Consistent with this, cell fractionation assay showed that more cytochrome c was released from mitochondria to cytosol in cells overexpressing the truncated proteins (Figure 5B). These results indicated that both forms of the cleaved Bcl-2 fragments, ΔN31 and ΔN34, increased the sensitivity of cells to apoptotic stimuli.

## Discussion

It was previously reported that Bcl-2 could be cleaved under apoptotic stimuli, which can inactivate its anti-apoptotic activity or even convert Bcl-2 to a Bax-like effector [11, 12]. It was proposed that Bcl-2 cleavage could act as a positive feedback amplification loop that accelerates the apoptotic process [25, 26]. In the course of examining the transformation of Bcl-2 protein, we found two cleaved fragments around 23 kDa that were recognised by an anti-human Bcl-2 mAb against an epitope within amino acids 41-54 (Figure 3A). It was reported that $Asp^{34}$ was the cleavage site in Bcl-2 for caspase-3 and $Asp^{31}$ was also required for this cleavage [12], which was supported by our point-mutation experiments (Figure 3B). It was consistent with the fact that caspase-3 preferentially cleaves substrates containing aspartic acid residues at both P1 and P4 positions (DXXD↓).

Our present studies also showed that a larger fragment (ΔN31) was cleaved from the Bcl-2-D34A mutant in the presence of cisplatin (Figure 3B, panel D34A), suggesting other caspases that do not need the cleavage site of $Asp^{34}$ were also involved in the drug-induced cleavage of Bcl-2. Besides caspase-3, other caspases such as caspase-2, -6, -7, -8, -9 and -10 also participate in the apoptotic process [19]. Since we have previously shown a caspase-8 mediated cleavage of Bcl-2, which was independent of caspase-3 [16], we proposed that $Asp^{31}$ may be the potential caspase-8 cleavage site. In support of this, the generation of ΔN31 was eliminated when caspase-8 activity was inhibited either by the caspase-8 specific inhibitor (Figure 4A) or by the dominant-negative caspase-8 (Figure 4C). Further experiments also supported this conclusion since the larger cleaved fragment of Bcl-2 was produced in MCF-7 cells that lack caspase-3 activity [14], and its production was inhibited by the caspase-8 specific inhibitor (Figure 4B). The coactions by two different caspases may accelerate the process of Bcl-2 cleavage and rapidly amplify apoptotic signals.

There are several protein bands around 32.5 kDa detected by Western-blotting in cisplatin-treated cells (Figure 2A, 3B and 4A), among which some were faintly visible in cells expressing the D31,34A double mutant of Bcl-2 (Figure 3B) or in cells treated with caspases inhibitors (Figure 4A). They might be produced by cleavages at certain aspartic acid residues within EGFP that satisfy the recognition sites of certain caspases. Accompanied with the reduction of apoptosis, the activation of caspases would also be inhibited by overexpressing the D31,34A mutant, which would result in weakening of these intermediate bands. Another possible explanation is that these bands are due to nonspecific recognition by the Bcl-2 antibody used in these experiments since these bands were also seen in some cells without cisplatin treatment (Figure 2B, lane1 and 3; Figure 4A, lane 1).

The Bcl-2 protein loses its fourth region of homology domain (BH4) when cleaved at the loop domain. The BH4 domain, conserved among anti-apoptotic Bcl-2 family members, is required for homodimerization or heterodimerization with other apoptotic effectors [13, 27]. We transfected different truncated Bcl-2 constructs (ΔN31, ΔN31D34A, ΔN34) into CHO cells. All of them promoted the process of drug-induced apoptosis (Figure 5C), indicating that BH4 domain is crucial for the protective activity of Bcl-2. Removing BH4 domain may lead to the exposure of the pro-apoptotic BH3 domain of Bcl-2, reminiscent of the cleavage on Bid [28]. The truncated Bcl-2 facilitates the release of cytochrome c (Figure 5B). The cytosolic cytochrome c together with Apaf-1 activates caspase-9, which in turn activates caspase-3 [29]. Thus the caspase cascade could be amplified by the cleavage of Bcl-2.

## Acknowledgment

This work was supported by grants of National Natural Science Foundation of China (#30230110, #0637S12442, #30670433) to Jiarui Wu.

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