The phosphorylation status and anti-apoptotic activity of Bcl-2 are regulated by ERK and protein phosphatase 2A on the mitochondria

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Abstract Bcl-2 protein play important roles in the regulation of apoptosis. We previously reported that the phosphorylation of Bcl-2 was augmented by treatment with protein phosphatase 2A (PP2A) inhibitor; however, the kinase responsible for Bcl-2 phosphorylation had not yet been identified. In this study, we identified extracellular-signal-regulated kinase (ERK) as the responsible kinase for the phosphorylation of Bcl-2. We also found that the transmembrane region (TM) deleted form of Bcl-2 (Bcl-2ΔTM), which was unable to localize on the mitochondria was constitutively phosphorylated, whereas wild-type Bcl-2 that localized on the mitochondria, was present in its hypophosphorylated form. The phosphorylation of Bcl-2ΔTM was retarded by treatment with MAP kinase ERK kinase (MEK) inhibitor and PP2A did not bind to Bcl-2ΔTM. These observations suggest that Bcl-2ΔTM is constitutively phosphorylated by ERK, but is not dephosphorylated by PP2A in human tumor cell lines. The phosphorylation of Bcl-2 resulted in a reduction in anti-apoptotic function, implying that dephosphorylation promoted the anti-apoptotic activity of Bcl-2 protein in human tumor cell lines. Thus, the present findings suggest that ERK and PP2A are physiological regulators of Bcl-2 phosphorylation, and these enzymes exert an influence on the anti-apoptotic function of Bcl-2.
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Keywords: Apoptosis; Bcl-2; Protein phosphatase 2A; Extracellular-signal-regulated kinase; Phosphorylation

1. Introduction

Apoptosis is a physiological cell suicide mechanism that is important for the homeostasis and development of multicellular organisms [1]. Thus, the dysregulation of apoptosis contributes to many diseases, such as cancer, AIDS, and neuronal disease. The Bcl-2 family proteins are key regulators of apoptosis that have been classified into two groups, namely, anti-apoptotic members (Bcl-2, Bcl-X_L and Mcl-1) and pro-apoptotic members (Bax, Bad, Bid and Bcl-X_S). The *bcl-2* gene encodes a 26-kDa mitochondrial membrane protein that inhibits apoptosis induced by various stimuli and is overexpressed in many human tumors [2–5]. Many interpretations of Bcl-2 functions have been proposed such as inhibition of cytochrome *c* release from the mitochondria [6] and regulation of calcium flux [7].

Signal transduction mechanisms in eukaryotic cells make great use of phosphorylation and dephosphorylation in order to promote the rapid and reversible modification of many proteins [8]. The activity of the mitogen-activated protein kinase (MAPK) family, which includes extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, are regulated by phosphorylation [9,10]. ERK are known to act as anti-apoptotic molecules by transducing survival signals, whereas the activation of JNK or p38 results in the promotion of apoptosis [11]. Members of the MAPK family are thought to be crucial molecules for cell growth, differentiation, and apoptosis. Although several substrates of the MAPK family are already known, the identification of additional substrates will enhance our general understanding of the important roles played by the MAPK family.

Bcl-2 was found to be phosphorylated after tubulin damage [12] and during the M phase in the normal cell cycle [13,14]. Several kinases have been proposed to be responsible for phosphorylating Bcl-2, such as protein kinase C (PKC), JNK, and cell division cycle 2 (cdc2) kinase [15–17]. Moreover, studies of Bcl-2 phosphorylation under physiological conditions in human colon, tonsil, and other tissues have been reported [18]. However, the role of Bcl-2 phosphorylation under physiological conditions remains poorly understood.

Previously, we reported that Bcl-2 was phosphorylated at the Ser-87 residue in normal human blood cells; however, in tumor cells, Bcl-2 was dephosphorylated by protein phosphatase 2A (PP2A) [19]. In this study, we demonstrated that (1) Bcl-2 was constitutively phosphorylated by ERK at the Ser-87 residue and is dephosphorylated by PP2A; (2) a mutant of Bcl-2 which could not localize on the mitochondria was constitutively phosphorylated at the Ser-87 residue by ERK; and (3) the localization of Bcl-2 on the mitochondria is required for the dephosphorylation of ERK-phosphorylated Bcl-2 by PP2A.

2. Materials and methods

2.1. Cell lines

Human tumor cell lines HepG2 (Hepatoma), HeLa (Cervical carcinoma), A431 (Epidermoid carcinoma), and A549 (Lung adenocarcinoma) were cultured in DMEM supplemented with 10% FCS at 37 °C

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Abbreviations: CPT, camptothecin; ERK, extracellular-signal-regulated kinase 1 and 2; JNK, c-Jun N-terminal kinase 1 and 2; MAPK, mitogen-activated protein kinase; MEK, MAP kinase ERK kinase; PKC, protein kinase C; PLM-F, phoslactomycin F; PP2A, protein phosphatase 2A; TM, transmembrane region; wt, wild-type

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in an incubator containing 5% CO_2. K562 cells (Erythroleukemia) were cultured in RPMI-1640 supplemented with 10% FCS at 37 °C in an incubator containing 5% CO_2.

2.2. Materials

Phoslactomycin F (PLM-F) (PP2A inhibitor) was isolated from *Streptomyces* sp. as described previously [20]. Calphostin C (PKC inhibitor), KT5720 (c-AMP dependent protein kinase inhibitor), rapamycin (S6 kinase inhibitor), PD98059 (MAP kinase ERK kinase (MEK) inhibitor), and U0126 (MEK and S6 kinase inhibitor) were purchased from Calbiochem (San Diego, CA). Camptothecin (CPT, DNA topoisomerase I inhibitor) and wortmannin (phosphatidylinositol-3 kinase (PI-3 kinase) inhibitor) were obtained from Sigma (St. Louis, MO). SP600125 (JNK inhibitor) was purchased from BIOMOL (Plymouth Meeting, PA). SB202190 (p38 inhibitor) was a gift from Dr. T. Sudo.

2.3. Plasmids

Human full-length *bcl-2* cDNA or mutant-types of *bcl-2* cDNAs were subcloned into a pCI-neo vector (Promega, Madison, WI). Human full-length *ERK2* cDNA was subcloned into the pZeoSV2(+) vector (Invitrogen, Carlsbad, CA). The nucleotide sequences were checked by the dideoxynucleotide-chain-termination procedure using an automated sequencer (Applied Biosystems, Foster City, CA).

2.4. Establishment of stable Bcl-2 overexpressing cell lines

The HepG2 cells were transfected with wild-type Bcl-2 (Bcl-2wt) or mutant forms of Bcl-2-expression vectors using FuGENE 6 transfection reagent (Roche Diagnostics AG, Basel, Switzerland), which was followed by G418 selection. The cells transfected with the pCI-neo vector were designated as HepG2-Neo cells. The transmembrane region (TM)-deleted Bcl-2 (Bcl-2ΔTM) lacks a.a. 219–239 of Bcl-2 [21].

2.5. Western blot analysis

Cells were lysed in lysis buffer (10 mM HEPES, 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% Nonidet P-40, 0.1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.2) at 4 °C. To detect the mobility shifts in the Bcl-2 protein due to phosphorylation, a SDS–polyacrylamide gel containing 15% acrylamide and 0.07% *N*,*N*′-methylenebisacrylamide was used for separation. Proteins were immunoblotted with anti-Bcl-2 antibody (mouse monoclonal, DAKO, Glostrup, Denmark), anti-α-tubulin antibody (mouse monoclonal, Sigma), anti-pS87-Bcl-2 antibody (rabbit polyclonal, Oncogene), anti-ERK2 antibody (mouse monoclonal, BD Biosciences, San Jose, CA), and anti-PP2A/C antibody (rabbit polyclonal, Calbiochem).

2.6. In vitro kinase assay

Recombinant active ERK2 was purchased from Calbiochem. His_6-tagged recombinant Bcl-2, Bcl-2-S70A, Bcl-2-S87A, and Bcl-2-S70AS87A that lacked the C-terminal TM domain were expressed in *Escherichia coli*. BL21 (DE3) cells and the proteins were purified using Ni-nitrilotriacetic acid affinity agarose (Qiagen, Hilden, Germany). Removal of the TM domain was necessary to produce soluble recombinant proteins. The kinase assay was carried out by placing the recombinant proteins in reaction buffer (50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 10 μCi [γ-^{32}P] ATP, and 20 μM unlabeled ATP) at 30 °C for 30 min. The samples were resolved by SDS–PAGE and visualized by autoradiography.

2.7. Immunoprecipitation

The cells were lysed in TENSV buffer (50 mM Tris–HCl, 100 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, and 1% Nonidet P-40, pH 7.5). One-milligram aliquots of cell lysate were incubated with anti-Bcl-2 antibodies (Santa Cruz Biotechnology) at 4 °C for 2 h. The immune complexes in the cell lysate were precipitated by centrifugation. The resultant pellets as well as the whole cell lysates were subjected to SDS–PAGE, immunoblotting, and detection with either anti-PP2A/C (rabbit polyclonal, Calbiochem) or anti-ERK2 (mouse monoclonal, BD Biosciences) antibodies.

2.8. Fluorescence microscopy

Cells grown on coverslips were treated with 200 nM MitoTracker Red (Molecular Probes, Eugene, OR) for 30 min and then were fixed with 3.7% formaldehyde in PBS. After washing the cells with PBS, they were incubated in 0.1% Triton X-100 in PBS for 5 min, washed once with PBS, and incubated with anti-Bcl-2 antibody for 30 min. Alexa 488 conjugated anti-mouse IgG (Molecular Probes) was used as the secondary antibody. After washing the samples for an additional three times, they were incubated with 10 μg/ml Hoechst 33258 (Wako, Osaka, Japan) for 5 min in the dark in order to stain the nuclei. The cells were washed 3 more times with PBS and were subsequently examined using a fluorescence microscope (Olympus, Tokyo, Japan).

2.9. Phosphatase assay

Bcl-2ΔTM protein was immunoprecipitated with anti-Bcl-2 antibodies (rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) and incubated with alkaline phosphatase (2 U, Sigma) or PP2A (2 U, Calbiochem) in the presence or absence of phosphatase inhibitors (50 mM NaF, 2 mM sodium orthovanadate, 5 mM EDTA, and 5 mM EGTA). The resulting samples were electrophoresed and immunoblotted with anti-Bcl-2 antibody, as described above.

3. Results

3.1. ERK-mediated Bcl-2 phosphorylation at the Ser-87 residue after PP2A inhibition

To identify the kinase responsible for the phosphorylation of Bcl-2 after PLM-F treatment, we treated Bcl-2 overexpressed HepG2 cells (HepG2-Bcl-2wt) with PLM-F in the presence of 8 different kinase inhibitors. Treatment with MEK inhibitor, PD98059, or U0126 reduced the level of PLM-F-induced Bcl-2 phosphorylation, as visualized by a band shift from the slower to the faster migrating form of the protein detected by Western blot analysis (Fig. 1A). Treatment with MEK inhibitor, PD98059, or U0126 completely reduced phosphorylation level of ERK1/2, but not phosphorylation of p38 and JNK in our assay system (data not shown). However, treatment with 6 other kinase inhibitors (SB202190, SP600125, rapamycin, wortmannin, calphostin C, or KT5720) had little effect on phosphorylation level of Bcl-2 and ERK1/2 (Fig. 1A, and data not shown). Thus, the results suggested that PLM-F-induced Bcl-2 phosphorylation is mediated by the MEK-ERK pathway. We focused on ERK as a potential Bcl-2 kinase after PLM-F treatment, because ERK are downstream kinases in the MEK-ERK pathway, and the consensus sequence for the ERK-phosphorylated site of target proteins is PXn(S/T)P, where Xn is normally one residue in length, but can in some cases be two residues that are either neutral or basic in nature [22]. It has previously been demonstrated that the (65-PVARTSP-71) and (84-PALSP-88) sequences are both suitable targets for phosphorylation by ERK. Mutant Bcl-2 proteins (Bcl-2-S70A, Bcl-2-S87A, and Bcl-2-S70AS87A) were produced in the present study by *E. coli*. An in vitro kinase assay demonstrated that ERK phosphorylated Bcl-2, but ERK did not phosphorylate either Bcl-2-S87A or Bcl-2-S70AS87A (Fig. 1B). Thus, the findings indicated that ERK phosphorylates Bcl-2 at the Ser-87 residue. To test whether or not Bcl-2 can interact with ERK, co-immunoprecipitation experiments were performed using the extracts of cells transfected with human ERK and Bcl-2 or control vector. Immunoprecipitation of Bcl-2wt resulted in co-precipitation with ERK, indicating that these proteins bind to each other in situ (Fig. 1C). In the control experiments, no ERK protein was detected by co-precipitation from the extracts of cells that did not express Bcl-2. These observations suggested that ERK are associated with Bcl-2 and that they phosphorylate at the Ser-87 residue.

3.2. Characterization of Bcl-2ΔTM overexpressed HepG2 cells

ERK are known to localize in the cytoplasm and to translocate into the nucleus upon growth stimulation. In

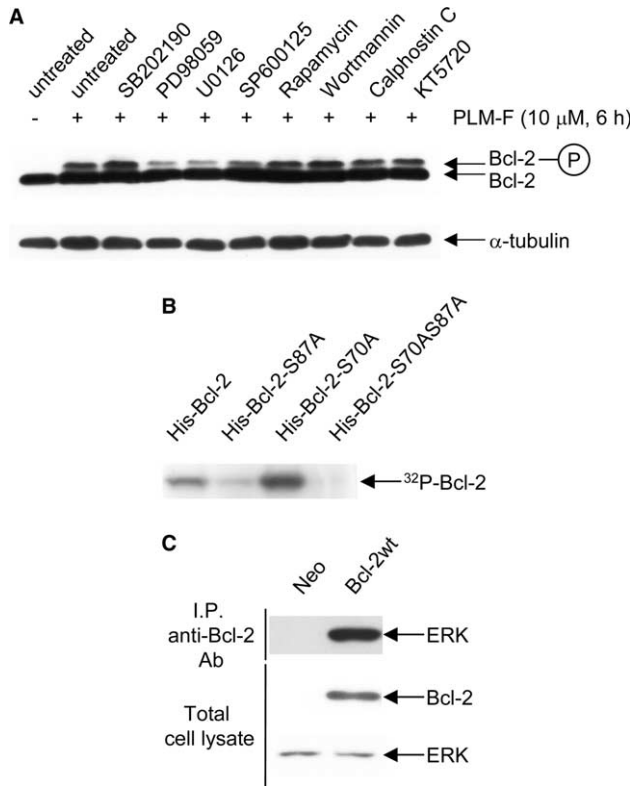

Fig. 1. ERK phosphorylates Bcl-2 at the Ser-87 residue. (A) The MEK inhibitors PD98059 and U0126 suppressed the phosphorylation of Bcl-2 induced by PLM-F treatment. HepG2-Bcl-2wt cells were untreated (lane 1) or treated with 10 μM PLM-F for 6 h (lanes 2-10) and 8 kinase inhibitors (lanes 3–10); 10 μM SB202190 for 2 h (lane 3), 10 μM PD98059 for 2 h (lane 4), 10 μM U0126 for 2 h (lane 5), 10 μM SP600125 for 2 h (lane 6), 100 nM rapamycin for 6 h (lane 7), 100 nM wortmannin for 6 h (lane 8), 100 nM calphostin C for 6 h (lane 9), or 100 nM KT5720 for 6 h (lane 10). The cells were lysed and aliquots of the cell lysates were analyzed by Western blotting using anti-Bcl-2 and anti-α-tubulin antibodies. (B) The Ser-87 residue of Bcl-2 is phosphorylated by ERK in vitro. An in vitro kinase assay was carried out using active recombinant ERK and Bcl-2, Bcl-2-S87A, Bcl-2-S70A, or Bcl-2-S70AS87A proteins. Only Bcl-2 and Bcl-2-S70A were phosphorylated to a significant level. (C) Association of ERK with Bcl-2 in situ. HepG2-Neo or HepG2-Bcl-2wt cells were transiently transfected with pZeo-*ERK2* plasmid for 24 h, followed by lysis and immunoprecipitation (IP) with anti-Bcl-2 antibody. Non-immunoprecipitated total cell lysates were analyzed in parallel.

contrast, Bcl-2 is anchors on the mitochondrial outer membrane through the C-terminal TM [21]. The localization of these proteins is somehow a contradiction. We examined whether or not mutant Bcl-2, which could not localize on the mitochondria, is phosphorylated. To this end, we established TM-deleted Bcl-2 (Bcl-2ΔTM) overexpressing HepG2 cells (designated as HepG2-Bcl-2ΔTM cells) (Fig. 2A). Immunocytochemistry of Bcl-2 overexpressing cells revealed the distribution of the protein on the mitochondria. In contrast, the merge panel of Bcl-2ΔTM overexpressing cells indicated that the distribution of Bcl-2ΔTM is not only in cytosol but also in nucleus and mitochondria (Fig. 2B). To examine whether Bcl-2ΔTM is localized on mitochondria, we purified mitochondria from Bcl-2ΔTM-expressing cells, and carried out western blotting analysis for detection of Bcl-2ΔTM protein. The data revealed that Bcl-2ΔTM is not localized at mitochondria any more (data not shown). This phenomenon is consistent with the previous report [21]. Moreover, our data clearly indicate that Bcl-2ΔTM was localized in the nucleus. Indeed, it was reported that phosphorylated form of Bcl-2 was detected in chromosome [23]. Thus, Bcl-2ΔTM was localized at cytosol and nucleus, but not mitochondria. Interestingly, Bcl-2wt was detected as a single band in the extracts of HepG2-Bcl-2wt cells; however, in the extracts of HepG2-Bcl-2ΔTM cells, two forms of Bcl-2ΔTM were clearly observed (Fig. 2C). Since the slower migrating form of Bcl-2ΔTM is thought to be a phosphorylated-form of Bcl-2ΔTM, we treated it with alkaline phosphatase in vitro. As shown in Fig. 2D, treatment with alkaline phosphatase eliminated the slower migrating form and gave only a faster migrating form, and this alteration was retarded by phosphatase inhibitor treatment, suggesting that some of the Bcl-2ΔTM is constitutively phosphorylated.

3.3. Phosphorylation of Bcl-2ΔTM at the Ser-87 residue by ERK in situ

Based on the results of a sitespecific point mutant study and Western blot analysis using anti-pS87-Bcl-2 antibody, we revealed that Bcl-2ΔTM is constitutively phosphorylated at the Ser-87 residue (Fig. 3A and B). In addition, similar results were observed in other human tumor cell lines, i.e., in HeLa, A431, A549, and K562 cells (Fig. 3C). We next tested whether the phosphorylation of Bcl-2ΔTM is also due to MEK-mediated regulation. As expected, the phosphorylation of Bcl-2ΔTM was inhibited by treatment with PD98059 or U0126 (Fig. 3D). Moreover, immunoprecipitation of Bcl-2wt protein resulted in co-precipitation with ERK, indicating that these proteins do interact in situ and that Bcl-2ΔTM does not quantitatively affect this interaction (data not shown). These results suggest that Bcl-2ΔTM is constitutively phosphorylated by ERK at the Ser-87 residue.

3.4. Association of Bcl-2 protein with PP2A on the mitochondria

To determine whether the phosphorylation of Bcl-2ΔTM is due to dissociation from PP2A, we carried out an immunoprecipitation assay. As shown in Fig. 4A, the immunoprecipitation of Bcl-2wt, but not that of Bcl-2ΔTM, resulted in the co-precipitation of PP2A. To confirm whether or not PP2A directly dephosphorylates Bcl-2 protein in vitro, we treated the cells with PP2A of the immunoprecipitated and heat-inactivated Bcl-2 protein, which is phosphorylated at the Ser-87 residue. As shown in Fig. 4B, PP2A dephosphorylates immunoprecipitated Bcl-2ΔTM; moreover, the dephosphorylation is canceled in the presence of PLM-F. Thus, Bcl-2 was dephosphorylated by PP2A in vitro, which suggests that the localization of Bcl-2 on the mitochondria is required for the dephosphorylation of Bcl-2 by PP2A.

3.5. Abrogation of the anti-apoptotic function of Bcl-2ΔTM by phosphorylation at the Ser-87 residue

Finally, we examined the effects of Bcl-2ΔTM phosphorylation at Ser-87 on anti-apoptotic function. Treatment of HepG2-Neo cells with 1 μg/ml CPT for 24 h led to apoptosis, whereas such drug-induced apoptosis was clearly reduced in HepG2-Bcl-2wt cells. In approximately 40% of the HepG2-Bcl-2ΔTM cells, apoptosis was induced following CPT treatment; however, in HepG2-Bcl-2-S87AΔTM cells,

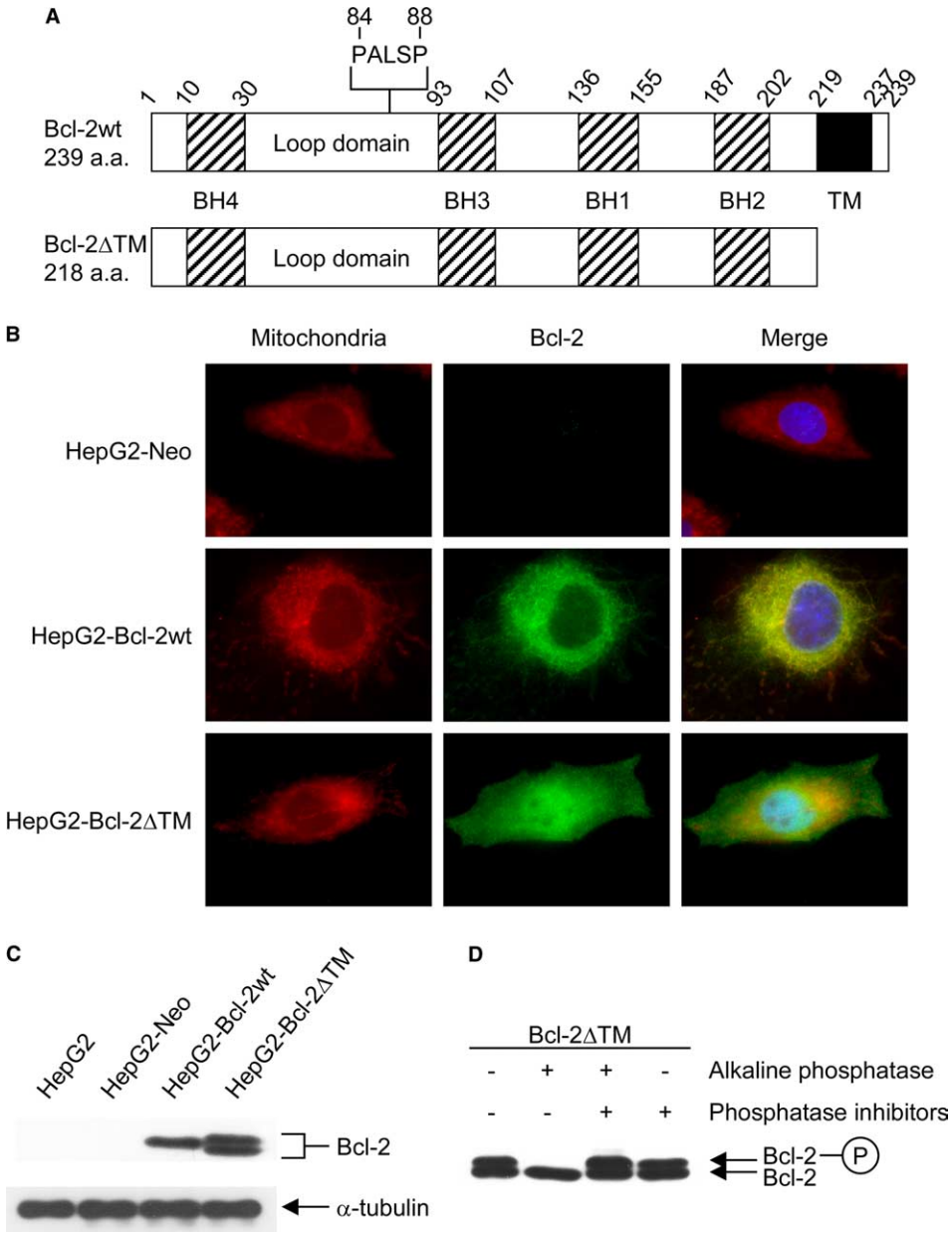

Fig. 2. Characterization of Bcl-2ΔTM overexpressed HepG2 cells. (A) Schematic diagram of the Bcl-2wt and Bcl-2ΔTM proteins. The TM (residues 219–237) is denoted by a black box and the Bcl-2 homology (BH) domains (residues 10–30 for BH4, residues 93–107 for BH3, residues 136–155 for BH1, and residues 187–202 for BH2) are designated by hatched boxes. (B) Subcellular localization of Bcl-2 and Bcl-2ΔTM proteins. Exponentially growing HepG2 cells transfected with pCI-neo, pCI-*bcl-2*, or pCI-*bcl-2*ΔTM were stained with Hoechst 33258 (for nuclei; blue), MitoTracker Red (for mitochondria; red), and anti-Bcl-2 antibody (green), and the cells were observed using fluorescence microscopy. (C) Expression of Bcl-2 and Bcl-2ΔTM proteins. Exponentially growing HepG2, HepG2-Neo, HepG2-Bcl-2wt, and HepG2-Bcl-2ΔTM cells were lysed and the total cell lysates were analyzed by Western blotting using anti-Bcl-2 and anti-α-tubulin antibodies. (D) Phosphorylation of Bcl-2ΔTM in situ. An exponentially growing cell line expressing Bcl-2ΔTM was lysed and incubated with alkaline phosphatase in the presence or absence of phosphatase inhibitors. The resulting samples were analyzed by Western blotting using anti-Bcl-2 antibody.

the number of dead cells was reduced, i.e., only 20% of the cells underwent apoptosis (Fig. 5). Thus, we concluded that the constitutive phosphorylation of Bcl-2ΔTM at the Ser-87 residue reduces the potency of its anti-apoptotic function.

4. Discussion

Bcl-2 was originally identified as the proto-oncogene involved in the t(14; 18) translocation in human follicular lymphoma [24]. Bcl-2 turned out to be an unusual oncogene in that rather than providing a proliferative advantage to the cell, it enhanced its capacity to survive under sub-optimal conditions. Previous observations have thus suggested that changes in the regulation of Bcl-2 might influence the course of tumorigenesis and/or in the acquisition of drug resistance by tumor cells. Indeed, the overexpression of Bcl-2 has been observed in several types of human tumors [25–27]. For example, it has been reported that a phosphorylated form of Bcl-2 occurs in human tissues such as the colon [18]; however,

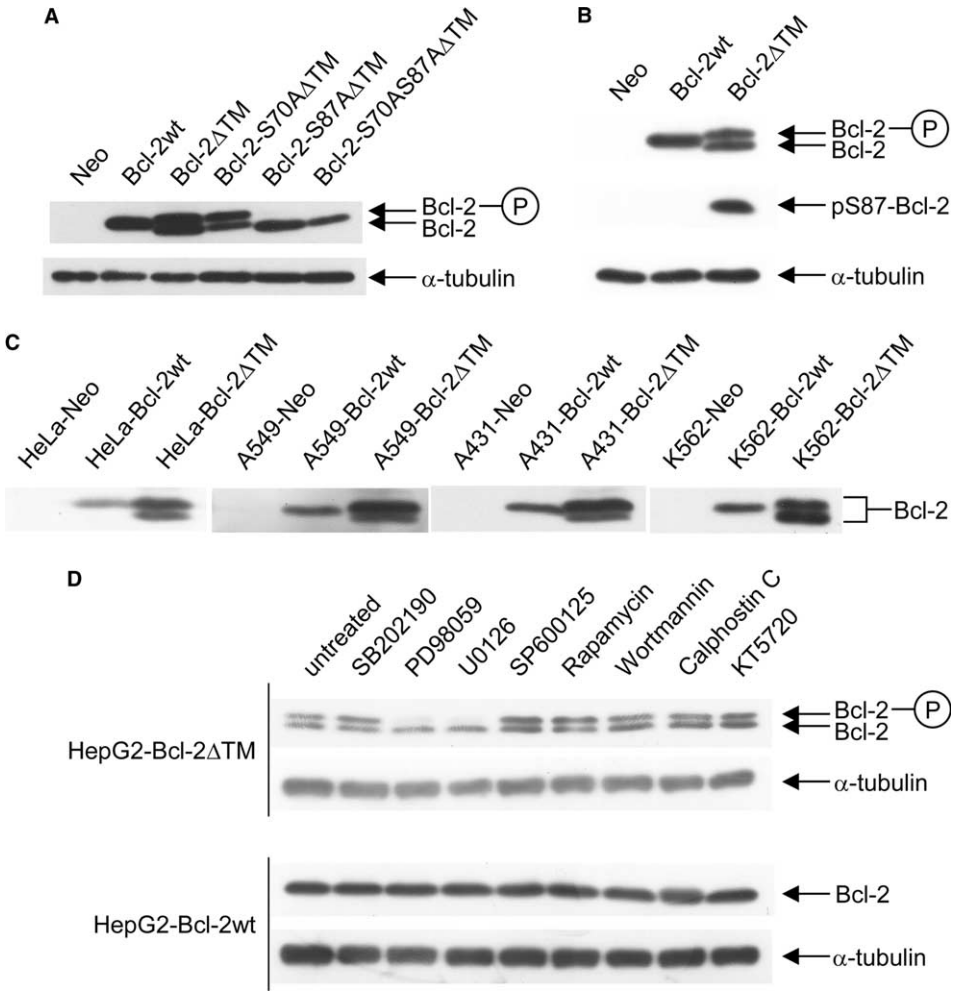

Fig. 3. Phosphorylation of Bcl-2ΔTM at the Ser-87 residue by ERK in situ. (A) and (B) Phosphorylation of Bcl-2ΔTM at the Ser-87 residue in situ. Exponentially growing cell lines expressing Bcl-2wt or mutant forms (Bcl-2ΔTM, Bcl-2-S70AΔTM, Bcl-2-S87AΔTM, or Bcl-2-S70AS87AΔTM) of Bcl-2 or carrying the control construct (Neo) were lysed and aliquots were analyzed by Western blotting using anti-Bcl-2 and anti-α-tubulin antibodies (A) and anti-pS87-Bcl-2 antibody (B). (C) Phosphorylation of Bcl-2ΔTM protein in several human tumor cell lines. Exponentially growing HeLa, A431, A549, and K562 cells were transiently transfected with pCI-neo, pCI-*bcl-2*, or pCI-*bcl-2*ΔTM. Total cell lysates were analyzed by Western blotting using anti-Bcl-2 antibody. (D) The MEK inhibitors PD98059 and U0126 suppressed the phosphorylation of Bcl-2ΔTM. HepG2-Bcl-2ΔTM (*upper*) or HepG2-Bcl-2wt (*lower*) cells were untreated (lane 1) or treated with 10 μM SB202190 for 2 h (lane 2), 10 μM PD98059 for 2 h (lane 3), 10 μM U0126 for 2 h (lane 4), 10 μM SP600125 for 2 h (lane 5), 100 nM rapamycin for 18 h (lane 6), 100 nM wortmannin for 18 h (lane 7), 100 nM calphostin C for 18 h (lane 8), or 100 nM KT5720 for 18 h (lane 9). The cells were lysed and aliquots of the cell lysates were analyzed by Western blotting using anti-Bcl-2 and anti-α-tubulin antibodies.

the physiological role of phosphorylation in this context is poorly understood.

Many interpretations of the purpose of Bcl-2 phosphorylation have been proposed; for example, it has been suggested that Bcl-2 phosphorylation is involved in the inactivation of anti-apoptotic function [12,16,28–30], marker of M phase events [31,32], and promotes anti-apoptotic mechanisms [33–36]. Here, we revealed that the expression of Bcl-2ΔTM, constitutively phosphorylated by ERK, demonstrated roughly comparable decreases in anti-apoptotic activity compared with Bcl-2-S87AΔTM (Fig. 5). These observations indicate that the phosphorylation of Bcl-2 results in the inactivation of anti-apoptotic function; thus, the present findings are consistent with those of previous reports [12,16,28–30]. However, our findings remain in contrast with the observations obtained by using a different system, in which unphosphorylated Bcl-2 displays less protection in certain cell lines [33–36]. Thus, it is likely that the regulation of the anti-apoptotic function of Bcl-2 by phosphorylation is dependent on cell-type specific factors that are as yet undefined.

We previously reported that the Ser-87 residue of Bcl-2 is constitutively phosphorylated in human normal blood cells and is dephosphorylated by PP2A [19]. Moreover, Ruvolo et al. [33,34] reported that Bcl-2 is dephosphorylated by mitochondrial PP2A in ceramide-mediated regulation. The findings of the present study suggest that ERK and PP2A co-regulate the status of Bcl-2 phosphorylation, without any external stimuli in human tumor cell lines. We identified the ERK as being Bcl-2ΔTM kinase (Figs. 1B and 3D). It has been proposed that ERK function as negative regulators of apoptosis by transducing survival signals [11]. In this study, we showed that ERK has the capacity to phosphorylate and inactivate the anti-apoptotic activity of Bcl-2, and thus they may act both as negative and as positive regulators of the

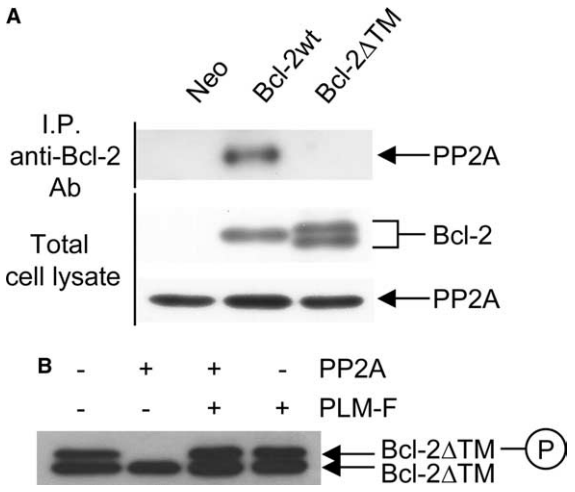

Fig. 4. Dephosphorylation of Bcl-2 by PP2A on the mitochondria. (A) Association of PP2A with Bcl-2wt, but not with Bcl-2ΔTM. HepG2-Neo, HepG2-Bcl-2wt, or HepG2-Bcl-2ΔTM cells were lysed and aliquots of the cell lysates were immunoprecipitated (IP) with anti-Bcl-2 antibody. In addition, non-IP cell lysates were analyzed by Western blotting. (B) Dephosphorylation of Bcl-2ΔTM by PP2A in vitro. Bcl-2ΔTM protein was immunoprecipitated with anti-Bcl-2 antibody, boiled for 3 min to quench the endogenous enzymes, and incubated with PP2A in the absence or presence of 100 μM PLM-F. The samples were analyzed by Western blotting using anti-Bcl-2 antibodies.

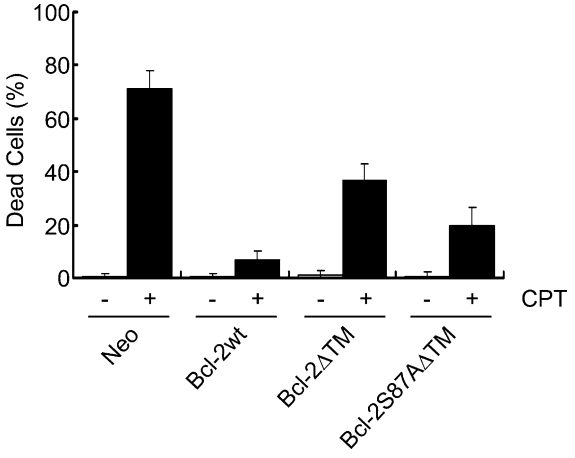

Fig. 5. Partial inhibition of CPT-induced apoptosis by Bcl-2ΔTM. The effects of Bcl-2, Bcl-2ΔTM, or Bcl-2-S87AΔTM overexpression on CPT-induced apoptosis. Each cell line was untreated (*open bars*) or treated (*solid bars*) with 1 μg/ml CPT for 24 h. Dead cells were assessed by trypan blue dye exclusion assay. Values are means ± S.D. of quadruplicate determinations.

induction of apoptosis. We demonstrated that ERK co-precipitate with Bcl-2 protein; these results are in agreement with those of Deng et al., who showed that ERK co-localize with Bcl-2 [37]. Moreover, the effect of MEK inhibitors completely depends on the conditions, MEK5 is usually more sensitive than MEK1/2 [38]. Thus, we could not exclude the possibility that PP2A inhibitor-induced Bcl-2 phosphorylation might be mediated by MEK1-ERK1/2 and/or MEK5-ERK5 pathways.

It has been reported that Bcl-2 is phosphorylated by unidentified kinase(s) after treatment with tubulin binder [12,28,29]. In this study, we demonstrated that the phosphorylation of Bcl-2wt by PLM-F treatment and the constitutive phosphorylation of Bcl-2ΔTM are mediated by ERK. Thus, we concluded that the ERK in this context are Bcl-2 kinases under physiological conditions in the absence of specific stimuli. On the other hand, the treatment of cells with PD98059 or U0126 failed to inhibit tubulin binder-induced Bcl-2 phosphorylation (data not shown). Therefore, it appears that the MEK-dependent pathway is not required for tubulin binder-induced Bcl-2 phosphorylation. Further study will be necessary in order to identify different Bcl-2 kinases and to clarify the role of Bcl-2 phosphorylation under different conditions, such as tubulin binder stimulation and the M phase of the normal cell cycle.

The present results, when taken together with those of our previous study, lead to the following three conclusions, which suggest a correlation between Bcl-2 phosphorylation status and tumorigenesis. (1) In normal human blood cells, Bcl-2 is phosphorylated at the Ser-87 residue; furthermore, the anti-apoptotic function of Bcl-2 might be inactivated by this mechanism [19]. (2) In human tumor cell lines, the Ser-87 residue of Bcl-2 is phosphorylated by ERK and this residue is immediately dephosphorylated by PP2A on the mitochondria. Thus, in human tumor cell lines, Bcl-2 is constitutively dephosphorylated and its anti-apoptotic function is activated. (3) Bcl-2ΔTM interacts with ERK, but it cannot interact with PP2A. Thus, Bcl-2ΔTM is constitutively phosphorylated by ERK and its anti-apoptotic function is weakened. Phosphorylation of Bcl-2ΔTM at the Ser-87 residue results in a decrease in anti-apoptotic function, whereas dephosphorylation activates the anti-apoptotic function in human tumor cell lines. These observations suggest that phosphorylation regulated by ERK and PP2A may have a significant impact on the oncogenic activity of Bcl-2.

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