

# Bcl-2 Phosphorylation by p38 MAPK

## IDENTIFICATION OF TARGET SITES AND BIOLOGIC CONSEQUENCES\*

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The antiapoptotic role of Bcl-2 can be regulated by its phosphorylation in serine and threonine residues located in a non-structured loop that links BH3 and BH4 domains. p38 MAPK has been identified as one of the kinases able to mediate such phosphorylation, through direct interaction with Bcl-2 protein in the mitochondrial compartment. In this study, we identify, by using mass spectrometry techniques and specific anti-phosphopeptide antibodies, Ser<sup>87</sup> and Thr<sup>56</sup> as the Bcl-2 residues phosphorylated by p38 MAPK and show that phosphorylation of these residues is always associated with a decrease in the antiapoptotic potential of Bcl-2 protein. Furthermore, we obtained evidence that p38 MAPK-induced Bcl-2 phosphorylation plays a key role in the early events following serum deprivation in embryonic fibroblasts. Both cytochrome *c* release and caspase activation triggered by p38 MAPK activation and Bcl-2 phosphorylation are absent in embryonic fibroblasts from p38 $\alpha$  knock-out mice (p38 $\alpha$ <sup>-/-</sup> MEF), whereas they occur within 12 h of serum withdrawal in p38 $\alpha$ <sup>+/+</sup> MEF; moreover, they can be prevented by p38 MAPK inhibitors and are not associated with the synthesis of the proapoptotic proteins Bax and Fas. Thus, Bcl-2 phosphorylation by activated p38 MAPK is a key event in the early induction of apoptosis under conditions of cellular stress.

Bcl-2 family proteins play differential roles in the regulation of mitochondria-mediated apoptosis, by either promoting or inhibiting the release of apoptogenic molecules from mito-

chondria to the cytosol (1, 2). Bcl-2 family proteins modulate the mitochondrial permeability through interaction with adenine nucleotide translocator, voltage-dependent anion channel, by ADP/ATP exchange, or by interfering with oxidative phosphorylation during apoptosis (2). The three-dimensional structure of Bcl-2 proteins suggests that they are also capable of forming channels in mitochondrial membranes (3–5).

Recent evidence indicates that the antiapoptotic functions of Bcl-2 can be regulated by post-translational modifications, including phosphorylation (6), dimerization (7), and/or proteolytic cleavage (8). There is still controversy over the significance of Bcl-2 phosphorylation, and two opposing models were initially proposed, the "taxol-induced" and the "interleukin-3-induced" Bcl-2 phosphorylation (6). In the first model, Bcl-2 phosphorylation induces a strong inhibition of the antiapoptotic potential and is related to hyperphosphorylation of several kinases, including Raf-1 kinase, but is independent of protein kinase C (9). In the second model, stimulation with growth factors, such as interleukin-3, induces Bcl-2 phosphorylation, which increases the antiapoptotic potential of the protein (10), whereas, in contrast, interleukin-3 deprivation inhibits Bcl-2-phosphorylation and induces cell death. However, subsequent reports clearly show that hyperphosphorylated Bcl-2 cannot be considered a hallmark of the growth/survival mechanism of action and that triggering specific tyrosine kinase receptors decreases Bcl-2 phosphorylation still inhibiting cell death (6). For example, binding of insulin receptor substrate proteins to Bcl-2, triggered by insulin/insulin-receptor interaction, inhibits its phosphorylation and has been proposed as one of the survival mechanisms induced by insulin (11). In memory B lymphocytes, as well as in continuous B lymphoblastoid cell lines, nerve growth factor (NGF)<sup>3</sup> inhibits apoptosis by preventing

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<sup>3</sup> The abbreviations used are: NGF, nerve growth factor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryo fibroblast(s); MDCK, Madin-Darby canine kidney; GST, glutathione S-transferase; PBS, phosphate-buffered saline; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; rp38, recombinant p38; WT, wild type; MS, mass spectrometry; ERK, extracellular signal-regulated kinase; PARP, poly-(ADP-ribose) polymerase.

## p38 MAPK and Bcl-2 Phosphorylation

Bcl-2 phosphorylation (12–14). Probably, both phenomena are not necessarily mutually exclusive and may reflect the function of Bcl-2 in different cellular contexts, able to induce directly or indirectly Bcl-2 phosphorylation. An alternative explanation is that such phenomena simply reflect the action of different Bcl-2 kinases, which phosphorylate different residues of the protein.

In fact, Bcl-2 phosphorylation occurs in at least one of the four serine and threonine residues located in a nonstructured loop that links the BH3 and BH4 domains (15), and different kinases and phosphatases have been reported to modulate such phosphorylation (16–19). JNK was repeatedly indicated as a potential Bcl-2 kinase, and it has been shown to induce Bcl-2 phosphorylation at four serine/threonine sites (Thr<sup>56</sup>, Ser<sup>70</sup>, Thr<sup>74</sup>, and Ser<sup>87</sup>) (19). However, recent reports suggest that, although paclitaxel-induced JNK activation is crucial for Bcl-2 phosphorylation, other kinases, such as CDK1, are responsible for it (20). Consistently, during paclitaxel treatment, lack of synchrony between JNK activation (early event) and Bcl-2 phosphorylation (late event) has been reported (21).

p38 MAPK is reportedly involved in several apoptotic pathways, including those triggered by NGF withdrawal, tumor necrosis factor, and NO, all inducing Bcl-2 phosphorylation (12–14, 22, 23). Under these conditions, Bcl-2 hyperphosphorylation induced by p38 MAPK always leads to proapoptotic events, as indicated by the release of cytochrome *c* both in cellular systems and in “cell-free” experiments (14). In contrast to JNK, Bcl-2 phosphorylation is synchronous with p38 MAPK activation and is mediated by a direct interaction between Bcl-2 and the activated enzyme in the mitochondrial compartment, resulting in cytochrome *c* release and caspase activation. Cellular apoptosis, triggered a few hours after p38 MAPK activation, does not require *de novo* protein synthesis and can efficiently be blocked by p38 MAPK-specific inhibitors. On the whole, these data support the hypothesis that several kinases can phosphorylate specific Bcl-2 residues and evoke different biologic events, thus underscoring the need to assess precisely those residues.

In this study, we identified, by using mass spectrometry techniques and specific anti-phosphopeptide antibodies, Ser<sup>87</sup> and Thr<sup>56</sup> as the Bcl-2 residues phosphorylated by p38 MAPK. Consistently, substitution of these residues with alanine inhibits cytochrome *c* release from isolated mitochondria after the addition of activated p38 MAPK. We also showed that deletion of the p38 $\alpha$  MAPK gene strongly delays apoptosis induced by serum withdrawal in embryonic fibroblasts and that Bcl-2 phosphorylation induced by p38 MAPK plays a key role in the early induction of apoptosis following serum deprivation.

## EXPERIMENTAL PROCEDURES

**Cell Cultures**—Embryonic fibroblasts (MEF) from p38 $\alpha$  MAPK<sup>+/+</sup> and p38 $\alpha$  MAPK<sup>-/-</sup> embryos were kindly donated by Dr. Micheal Karin (University of California, San Diego) (24). MEF were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum; CESS and MDCK cell lines were purchased by ATCC (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum.

**Reagents**—Mouse anti-cytochrome *c* Abs were purchased from BD Pharmingen (clone 7H8.2C12 for Western blotting analysis; clone 6H2.B4 for immunofluorescence analysis); rabbit anti-p38 MAPK, anti-PARP, anti-actin, anti-Fas/CD95, anti-Bcl-2, anti-Bax Abs, and mouse anti-human Bcl-2 Abs were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); rabbit anti-phosphorylated p38 MAPK Abs were purchased from New England Biolabs; and the p38 MAPK inhibitor SB203580 was purchased from Calbiochem. Human recombinant active p38 MAPK-GST and JNK-GST were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Human recombinant Bcl-2-(1–205) and its mutants T56A and S87A were expressed as N-terminal GST fusion protein using pGEX-4T-1 vector in BL21 Gold cells (*Escherichia coli*). Recombinant protein was purified by resuspending 1-liter cell cultures in ice-cold phosphate-buffered saline (PBS) supplemented with 0.5 mM phenylmethylsulfonyl fluoride protease inhibitor and 1 mM dithiothreitol. The cell suspension was incubated with 1 mg/ml lysozyme for 30 min at 4 °C, sonicated to reduce viscosity, and then mixed gently with 1% Triton X-100 for 30 min at 4 °C and centrifuged at 10,000 × *g* for 30 min. The supernatant was applied to a glutathione-Sepharose 4B column (Amersham Biosciences) and purified using a wash step with PBS until the A<sub>280</sub> reached <0.001. Proteins were eluted with 50 mM reduced glutathione in 200 mM Tris-HCl, pH 8, supplemented with 1 mM dithiothreitol, desalted against 50 mM Tris-HCl, pH 8, 1% glycerol, and stored at –80 °C.

Construction of Bcl-2 full-length mutants was performed as described (14). The T56A/S87A Bcl-2 full-length double mutant was constructed by a mutagenesis reaction using a QuikChange<sup>TM</sup> site-directed mutagenesis kit (Stratagene) and the T56A Bcl-2 full-length mutant cloned in pIRES2-EGF vector (Clontech) as template. Bcl-2 mutant sequences were then subcloned in pQE60 vector (Qiagen) for the expression as C-terminal His<sub>6</sub>-tagged proteins in M15 cells (*E. coli*). T56A and S87A Bcl-2 mutant sequences (residues 1–205) were then subcloned in pGEX4T1 vector for expression as N-terminal GST fusion protein in BL21 Gold cells (*E. coli*) as described above.

Anti-phospho-Bcl-2 (Thr<sup>56</sup> and Ser<sup>87</sup>) antibodies were produced in rabbits using as immunogens the synthetic peptides SSQPGH<sub>p</sub>TPHPAA and AAGPAL<sub>p</sub>SPVPPVV designed on the sequences 50–61 and 81–93 of human Bcl-2, in which pT and pS correspond to the phosphorylated threonine at residue 56 and serine at residue 87 of Bcl-2, respectively. The resulting antiserum was purified as described (25).

**Mass Spectrometry Analysis**—A kinase assay was performed by incubating Bcl-2-GST (20–30  $\mu$ g) in the absence (untreated sample) or presence (treated sample) of activated p38 MAPK in kinase buffer (50 mM Tris-HCl, 100 mM NaCl, 25 mM  $\beta$ -glycerophosphate, 25 mM MgCl<sub>2</sub>, pH 7.4, protease and phosphatase inhibitor mixtures, and 200  $\mu$ M ATP) for 30 min at 30 °C. Both treated and untreated samples were resolved on SDS-PAGE, and Bcl-2-GST bands were excised from gels and then subjected to overnight in-gel digestion with 12.5 ng/ $\mu$ l sequencing grade trypsin (Promega) at 37 °C as described (26, 27). Bcl-2-GST-digested peptides were recovered from gels, lyophilized, and then analyzed by mass spectrometry.

For MALDI time-of-flight (TOF) analysis, mass spectra were acquired in positive and negative ion mode using an Applied Biosystems/Sciex Q-Star XL instrument. All of the experiments were carried out with an oMALDI 2 ion source (oMALDI-QqTOF), setting the  $N_2$  laser power to 20  $\mu$ J with a pulse rate of 20 Hz.

**Cytochrome *c* Release**—MDCK cells, detached by trypsin-EDTA, were washed twice in phosphate-buffered saline and suspended in 8 ml of mitochondrial isolation buffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 20 mM  $MgCl_2$ , 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride protease inhibitor, 2  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, pH 7.4). Cells were passed through an ice-cold cylinder cell homogenizer. Unlysed cells and nuclei were pelleted via a 10-min,  $750 \times g$  spin. The supernatant was spun at  $10,000 \times g$  for 30 min at 4 °C, and the pellet containing the mitochondrial fraction was incubated for 30 min on ice and 15 min at 22 °C, with or without extracts of *E. coli* cells (8.5%, v/v), expressing native or mutated Bcl-2-His<sub>6</sub> or transformed with the empty plasmid, obtained by lysing the cells with 20 mM Hepes, pH 7.4, 30 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride protease inhibitor, 20  $\mu$ g/ml aprotinin, 20  $\mu$ g/ml leupeptin, and 1% CHAPS. 0.25 ng of active p38 MAPK, with or without 25  $\mu$ M SB203580, was then added, and the phosphorylation reaction was performed in the presence of 200  $\mu$ M ATP (Amersham Biosciences). p38 MAPK-untreated mitochondria were also used as control. Mitochondrial mixture reactions were then centrifuged at  $4,000 \times g$  for 10 min, and the supernatant and pellet proteins were separated on SDS-PAGE, followed by transfer to a polyvinylidene difluoride membrane (Amersham Biosciences). The blot was probed with anti-human cytochrome *c* peptide monoclonal antibody (7H8.2C12 clone; BD Biosciences), diluted to 1:500, followed by a secondary probe (1:5,000 dilution) with horseradish peroxidase-labeled antibodies (Amersham Biosciences). The reaction was visualized by ECL (Amersham Biosciences).

**Immunoblotting Techniques**—Lysates of p38 $\alpha^{+/+}$  MEF and p38 $\alpha^{-/-}$  MEF cells, grown under conditions of serum deprivation for different time periods, and lysates of CESS cells, cultured in the presence of 200 nM K252A (Calbiochem) as apoptotic stimuli for 2 h, were separated on SDS-PAGE and blotted on a polyvinylidene difluoride membrane (Amersham Biosciences). The blots were stained with the indicated primary antibodies (1  $\mu$ g/ml) followed by secondary probes (1:5,000 dilution) with horseradish peroxidase-labeled antibodies (Amersham Biosciences). The reaction was visualized by ECL (Amersham Biosciences).

**Kinase Assay**—For detection of Bcl-2 phosphorylation sites, a kinase assay was performed by incubating 0.25  $\mu$ g of recombinant active p38 MAPK-GST or active JNK-GST and 0.25  $\mu$ g of recombinant Bcl-2-GST in kinase buffer (25 mM Hepes, 25 mM  $\beta$ -glycerophosphate, 25 mM  $MgCl_2$ , 2 mM dithiothreitol, 0.1 mM orthovanadate, 200  $\mu$ M ATP) for 30 min at 30 °C in the presence or absence of 25  $\mu$ M SB203580 (Calbiochem). In another set of experiments, the kinase assay was performed by incubating 0.5  $\mu$ g of recombinant Bcl-2-GST or T56A Bcl-2-GST or S87A Bcl-2-GST and 0.25  $\mu$ g of recombinant active p38 MAPK-GST in kinase buffer for 30 min at 30 °C. p38 MAPK-

untreated Bcl-2 proteins were used as controls. Protein mixtures were separated by SDS-PAGE, blotted onto a nitrocellulose filter, and stained with anti-phospho-Bcl-2 (Thr<sup>56</sup> and Ser<sup>87</sup>) antibodies. The reaction was visualized by an ECL detection system (Amersham Biosciences).

To assess the activity of recombinant enzymes, 0.25  $\mu$ g of active rp38 MAPK-GST or recombinant JNK-GST were incubated with 0.25  $\mu$ g of rATF-2-GST in kinase buffer for 30 min in the presence of 3  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. Samples were run on SDS-PAGE and autoradiographed.

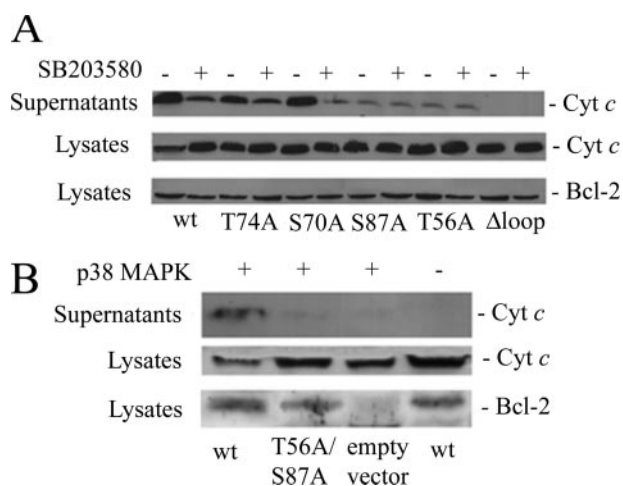
**Immunofluorescence Analysis**—For immunofluorescence analysis, p38 $\alpha^{+/+}$  and p38 $\alpha^{-/-}$  MEF were seeded on glass coverslips in 35-mm plates at a concentration of  $7 \times 10^4$  cells/glass; 24 h later, cells were washed twice with Dulbecco's modified Eagle's medium and then incubated in Dulbecco's modified Eagle's medium without serum for different times (6, 9, and 12 h). At the indicated time points, cells were washed twice in PBS, fixed for 30 min at 4 °C in 4% formaldehyde, incubated with 0.25  $\mu$ g/ml 4',6-diamidino-2-phenylindole for 10 min at 22 °C, and then permeabilized for 3 min with 0.3% Triton X-100 in PBS. After incubation with 3% (v/v) bovine serum albumin in PBS for 30 min to block nonspecific binding, the cells were incubated with anti-cytochrome *c* antibody (6H2.B4 clone; BD Pharmingen) in PBS plus 1.5% bovine serum albumin for 1 h at 37 °C in a humidified chamber. The coverslips were then rinsed three times in PBS and incubated with the secondary antibody, rinsed again in PBS, and examined by a Leica fluorescence microscope coupled to a CCD camera, equipped with a  $\times 100$  oil immersion lens.

## RESULTS

**p38 MAPK Phosphorylation of Thr<sup>56</sup> and Ser<sup>87</sup> Bcl-2 Residues Induces Release of Cytochrome *c* from Mitochondria: Biological Evidence and Relevance**—A variety of protein kinases, including the MAPK family members, have been reported to induce Bcl-2 phosphorylation (16–19). p38 MAPK was shown to induce Bcl-2 phosphorylation and apoptotic cell death in memory B lymphocytes upon withdrawal of their survival factor NGF (14). By transfecting lymphoid cells with Bcl-2 mutants in which each Thr<sup>56</sup>, Ser<sup>87</sup>, Ser<sup>70</sup>, and Thr<sup>74</sup> was substituted with alanine, we demonstrated that only T56A and S87A Bcl-2 mutants protected cells from apoptosis induced by p38 MAPK activation, thus indicating these sites as possible targets of the kinase (14).

In order to obtain further functional evidence that p38 MAPK-mediated phosphorylation of the Ser<sup>87</sup> and Thr<sup>56</sup> residues of Bcl-2 inhibits the antiapoptotic function of the protein, we produced the above reported Bcl-2 mutants in recombinant form and tested their function on isolated mitochondria from MDCK, a canine cell line that does not express Bcl-2 (28). Mitochondria, isolated as described (14), were incubated with recombinant Bcl-2 mutants, with recombinant Bcl-2 deprived of the entire loop region between the  $\alpha 1$  and  $\alpha 2$  helices (Bcl-2  $\Delta$  loop), or Bcl-2 wild type (Bcl-2 WT), as negative and positive controls, respectively. Following insertion of recombinant proteins in the membranes, activated p38 MAPK was added to the reaction mixture, and the release of cytochrome *c* was assessed as a measure of Bcl-2 antiapoptotic function.





**FIGURE 1. Effect of Bcl-2 loop region deletion or of T56A- and S87A-Bcl-2 mutations on cytochrome c release from mitochondria elicited by activated p38 MAPK.** Mitochondria isolated from MDCK cells were incubated with lysates of *E. coli* cells expressing Bcl-2-His<sub>6</sub> WT or the indicated mutants, in reaction buffer containing ATP and active p38 MAPK in the presence or absence of SB203580 (A). Mitochondria were also incubated with lysates of *E. coli* expressing Bcl-2-His<sub>6</sub> WT or T56A/S87A-Bcl-2-His<sub>6</sub> double mutant or lysates of *E. coli* transformed with the empty vector in reaction buffer containing ATP and, when indicated, active p38 MAPK (B). Supernatants of reaction mixtures and mitochondrial lysates were run on SDS-PAGE, blotted, and stained with anti-cytochrome c Abs. The mitochondrial extracts were immunoblotted also with anti-Bcl-2 Abs to ascertain the insertion of the recombinant proteins into the organelle membranes. Results from one experiment of three performed are shown. The densitometric analysis performed in three different experiments demonstrated levels of p38 MAPK-induced cytochrome c release from T56A-, S87A-, S70A- and T74A-bearing mitochondria of 17 ± 5, 15 ± 4, 88 ± 10, and 70 ± 8%, respectively, compared with the one from Bcl-2 WT-bearing mitochondria. Deletion of the Bcl-2 loop region or T56A or S87A single site mutations or the T56A/S87A double site mutations in this region inhibit cytochrome c release from mitochondria induced by activated p38 MAPK.

Fig. 1A shows that, after 2 h of incubation, p38 MAPK induced the release of cytochrome *c* from Bcl-2 WT-bearing mitochondria, and the phenomenon was inhibited by SB203580, a pyrimidyl imidazole-specific inhibitor of the enzyme (29). By contrast, the p38 MAPK-induced release of cytochrome *c* was not evident from mitochondria bearing the Bcl-2 Δloop and was strongly decreased from Bcl-2 S87A or Bcl-2 T56A mutants compared with that elicited from Bcl-2 WT-bearing mitochondria. Consistently, the Bcl-2 T56A/S87A double mutant-bearing mitochondria were completely resistant to p38 MAPK activity (Fig. 1B). Moreover, p38 MAPK failed to induce release of cytochrome *c* from mitochondria not exposed to Bcl-2, thus indicating that the Bcl-2/p38 MAPK interaction was causally related to the observed phenomenon, as previously demonstrated (14).

On the whole, the above experiments confirm that the substitution of Thr<sup>56</sup> and Ser<sup>87</sup> residues with alanine inhibits the cytochrome *c* release induced by p38 MAPK action on Bcl-2.

**p38 MAPK Phosphorylates Bcl-2 in Thr<sup>56</sup> and Ser<sup>87</sup> Residues: Biochemical and Immunochemical Demonstration**—To obtain a direct biochemical demonstration of the Bcl-2 residues phosphorylated by activated p38 MAPK, we performed a mass spectrometry analysis of tryptic fragments of Bcl-2 after phosphorylation by p38 MAPK *in vitro*.

A cell-free kinase assay was performed by incubating recombinant Bcl-2 with ATP in the presence or absence of

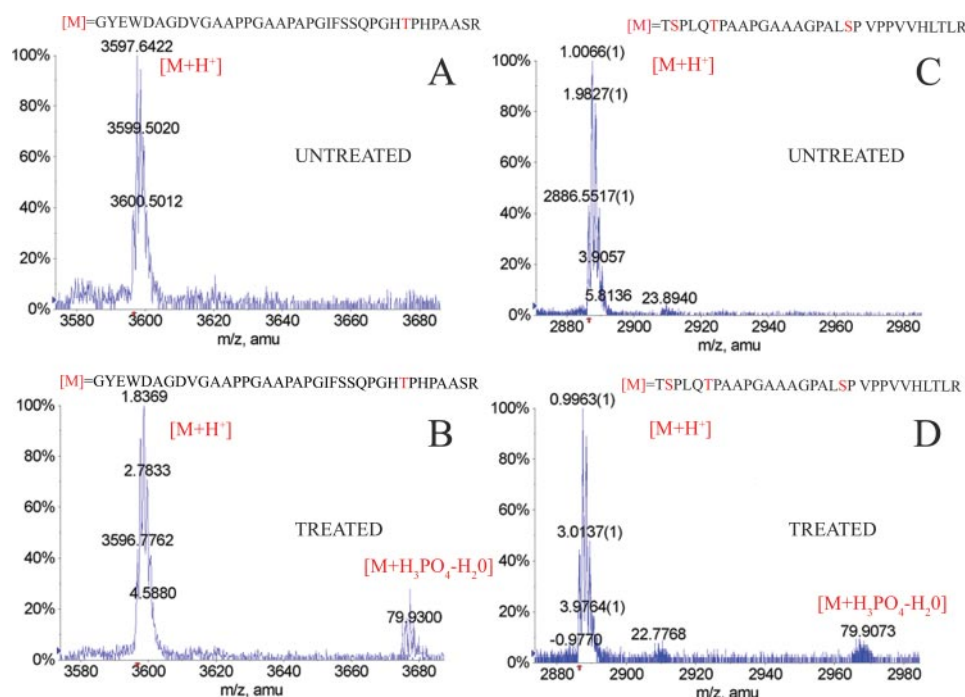
**TABLE 1**  
Peptides deriving from trypsin digestion of Bcl-2 protein

Peptide	Bcl-2 sequence	(M + H) <sup>+</sup> molecular weight
A	27–63	3,596.69
B	69–98	2,886.62

activated p38 MAPK. The reaction mixtures were separated on one-dimensional SDS-PAGE electrophoresis, and gel slices corresponding to phosphorylated or native Bcl-2 protein were excised and subjected to in-gel trypsin digestion. Digested peptides were extracted from gels, dried, and analyzed for mass differences by MALDI-TOF MS. As shown in Table 1, by monitoring the single charged ions of the two serine- and threonine-carrying fragments, two peptides, referred to hereafter as peptides A and B, respectively, were identified.

First, we focused our analysis on peptide A, since peptide B contains the three potentially phosphorylatable residues Ser<sup>70</sup>, Ser<sup>87</sup>, and Thr<sup>74</sup>, a condition not allowing the identification of the exact site phosphorylated by p38 MAPK. The unphosphorylated sample was used as negative control. Both the +80 mass (corresponding to the phosphate group) of the peptide A (as monoprotonated) and its phosphate fragment were detected in the p38 MAPK-phosphorylated sample. Fig. 2 shows enlarged portions, corresponding to peptide A mass range, of mass spectra of the peptide mixture from untreated Bcl-2 digest (Fig. 2A) and p38 MAPK-treated Bcl-2 digest (Fig. 2B). Both spectra presented a peak corresponding to unphosphorylated A peptide, which contains the Thr<sup>56</sup> residue as the only one within the MAPK consensus sequence (TP/SP). Indeed, only mass spectra of the peptide mixture from p38 MAPK-treated Bcl-2 showed a peak corresponding to phosphorylated A peptide, suggesting that the enzyme actually phosphorylates the residue Thr<sup>56</sup> of Bcl-2. Next, we analyzed the phosphorylation status of the B peptide. Fig. 2 shows enlarged portions, corresponding to the peptide B mass range, of mass spectra of the peptide mixture from untreated Bcl-2 digest (Fig. 2C) and p38 MAPK-treated Bcl-2 digest (Fig. 2D). As expected, both spectra presented a peak corresponding to unphosphorylated B peptide. Interestingly, the mass spectra of peptide mixture from p38 MAPK-treated Bcl-2 showed a peak corresponding to the monophosphorylated B peptide, indicating that the enzyme actually phosphorylates at least one residue among the three amino acids (Ser<sup>70</sup>, Ser<sup>87</sup>, and Thr<sup>74</sup>) lying within the MAPK consensus sequence. However, no signal corresponding to the double- or triple-phosphorylated peptides was detectable, suggesting that only one residue was the target of the enzyme. These results were confirmed when phosphopeptides were measured in negative ion mode (data not shown).

Thus, both A and B monophosphorylated peptides were unambiguously detected in the MALDI mass spectra. Nevertheless, the intensity of these peaks showed in Fig. 2 appeared low as compared with the unphosphorylated ones. This observation is consistent with the expected quenching effect elicited by the phosphate group on the MALDI ionization of the large hydrophobic A and B peptides. A similar phenomenon was observed when high pressure liquid chromatography-MS analysis of Bcl-2-GST tryptic peptides was performed (data not



**FIGURE 2. MALDI-TOF analysis of residue 27–63 and 69–98 Bcl-2 tryptic digested peptides obtained by untreated or p38 MAPK-treated Bcl-2 recombinant protein.** Recombinant Bcl-2-GST was incubated *in vitro* with ATP in the presence or absence of activated p38 MAPK. The reaction mixtures were separated on SDS-PAGE, and native (untreated sample) or p38 MAPK-phosphorylated (treated sample) Bcl-2 was subjected to in-gel tryptic digestion as described under "Experimental Procedures" and analyzed by mass spectrometry. MALDI-TOF spectra of Bcl-2 tryptic digested peptide 27–63 containing the potentially phosphorylatable residue Thr<sup>56</sup> (red in the sequence) from untreated sample (A) and treated sample (B) are shown. MALDI-TOF spectra of Bcl-2 tryptic digested peptide 69–98, containing the potentially phosphorylatable residues Ser<sup>70</sup>, Thr<sup>74</sup>, and Ser<sup>87</sup> (red in the sequence) from untreated sample (C) and treated sample (D) are shown. p38 MAPK phosphorylates the Thr<sup>56</sup> residue, and one among the Ser<sup>70</sup>, Thr<sup>74</sup>, and Ser<sup>87</sup> sites in the Bcl-2 loop region.

shown) using different analyzers (single quadrupole and tandem quadrupole TOF). For the same reasons, tandem mass sequence analysis failed to give the exact mapping of the phosphorylation sites.

Overall, the MALDI/MS analysis strongly indicated as targets of p38 MAPK enzymatic action the Thr<sup>56</sup> residue and one among the three potentially phosphorylatable residues Ser<sup>70</sup>, Ser<sup>87</sup>, and Thr<sup>74</sup>.

Since functional data (see Fig. 1 and Ref. 14) suggested Thr<sup>56</sup> and Ser<sup>87</sup> residues of Bcl-2 as the targets of the enzyme, to obtain direct evidence of their phosphorylation, we used a different experimental approach. Polyclonal antibodies against Thr<sup>56</sup>- or Ser<sup>87</sup>-phosphorylated sites of the protein were generated by immunizing rabbits with the synthetic phosphopeptides SSQPGHpTPHPAA and AAGPALpSPVPPVV, designed on the sequences 50–61 and 81–93 of human Bcl-2. Phosphorylated Bcl-2 proteins, obtained by an *in vitro* kinase assay with recombinant p38 MAPK (as reported above), were separated by SDS-PAGE electrophoresis, blotted on nitrocellulose membranes, and stained with anti-Bcl-2-specific phosphopeptide antibodies. Fig. 3A shows that, as expected, the anti-phospho-Thr<sup>56</sup>-Bcl-2 antibody stained the proteins after p38 MAPK enzymatic action, but not the native proteins. However, a phosphorylated Bcl-2 band was also revealed using an anti-phospho-Ser<sup>87</sup> antibody. Furthermore, SB203580 was able to inhibit phosphorylation of both Bcl-2 residues, thus confirming that they are targets of p38 MAPK enzymatic action.

An *in vitro* kinase assay performed with activated JNK as a phosphorylating enzyme showed no direct phosphorylation of the Thr<sup>56</sup> or Ser<sup>87</sup> residues of Bcl-2. However, both activated JNK and p38 MAPK were able to phosphorylate their common substrate ATF-2 (Fig. 3B).

In order to check the specificity of the anti-phospho-Bcl-2 antibodies and to definitely identify the target residues of p38 MAPK enzymatic action, a kinase assay was performed by incubating recombinant Bcl-2 T56A-GST or recombinant Bcl-2 S87A-GST mutants or, as a positive control, recombinant Bcl-2-GST with active p38 MAPK. Fig. 3C shows that the anti-phospho-Thr<sup>56</sup>-Bcl-2 antibody stained both the native protein and the S87A mutant, but not Bcl-2 T56A protein after p38 MAPK enzymatic action. On the contrary, the anti-phospho-Ser<sup>87</sup>-Bcl-2 antibody recognized Bcl-2 and T56A Bcl-2 proteins, but not the S87A mutant after the enzyme action. Moreover, both antibodies did not react with protein substrates not exposed to p38 MAPK. These results demonstrate

that the anti-phospho antibodies react strongly with their target phosphoresidues and only weakly, if at all, with other phosphoamino acids. Moreover, the results obtained with the anti-phospho-Bcl-2 antibodies definitively indicate Thr<sup>56</sup> and Ser<sup>87</sup> as the Bcl-2 residues phosphorylated by p38 MAPK.

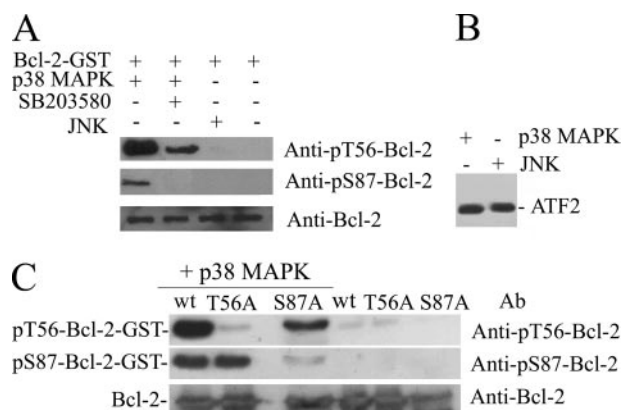
The same antibodies were used to detect phospho-Bcl-2 in CESS cells, a human B-cell line, in which activation of p38 MAPK induced by the TrkA inhibitor K252A results in Bcl-2 phosphorylation, with Ser<sup>87</sup> and Thr<sup>56</sup> being the most likely targets (14). Fig. 4 shows that both anti-phospho-Thr<sup>56</sup>- and anti-phospho-Ser<sup>87</sup>-Bcl-2 antibodies revealed a band of phosphorylated Bcl-2 protein, although the signal was much lower than that obtained with recombinant protein, probably due to the proteasomic degradation of the phosphorylated Bcl-2, as reported by others (30). As expected, phosphorylation of both residues was inhibited by SB203580 treatment, thus indicating Thr<sup>56</sup> and Ser<sup>87</sup> as the target of p38 MAPK action also in this cellular system.

Altogether, MALDI/MS analysis of tryptic Bcl-2 digests and specific anti-phospho antibody experiments contribute to demonstrate that the Thr<sup>56</sup> but also the Ser<sup>87</sup> Bcl-2 residues are specific targets of p38 MAPK enzymatic action.

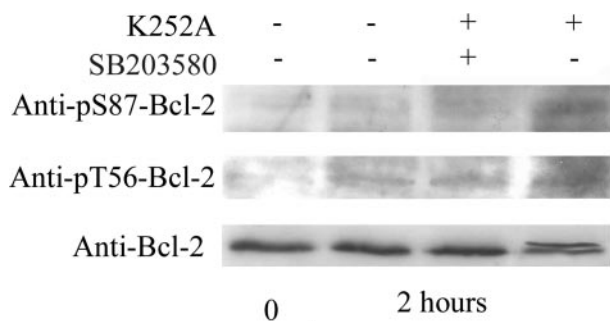
*Relevance of p38 MAPK-induced Bcl-2 Phosphorylation in Apoptotic Cell Death of MEF Induced by Serum Withdrawal*—The results reported above clearly show that p38 MAPK phosphorylation of the Thr<sup>56</sup> and Ser<sup>87</sup> Bcl-2 residues strongly alters the antiapoptotic function of the protein. In



## p38 MAPK and Bcl-2 Phosphorylation



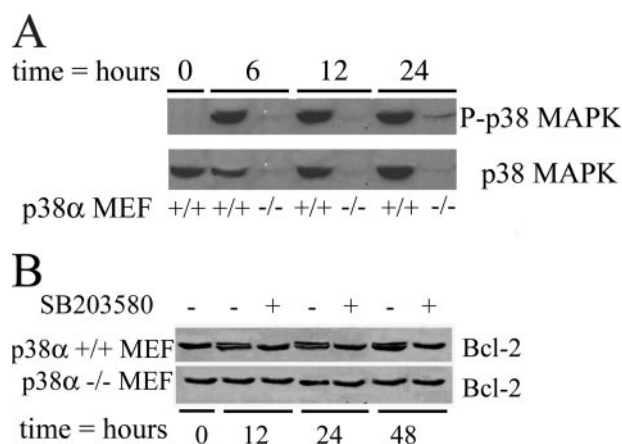
**FIGURE 3. Immunoblotting analysis of p38 MAPK-phosphorylated and JNK-phosphorylated Bcl-2 protein.** *A*, recombinant Bcl-2-GST was incubated in reaction buffer containing ATP and active rp38 MAPK-GST or active rp38 MAPK-GST plus SB203580 or active recombinant JNK-GST at 30 °C for 30 min. Reaction mixtures were run on SDS-PAGE, blotted, and stained with anti-phospho-Thr<sup>56</sup>-Bcl-2 Abs or anti-phospho-Ser<sup>87</sup>-Bcl-2 Abs. Reaction mixtures were immunoblotted also with anti-Bcl-2 Abs to ascertain the presence of the recombinant protein in each sample. Results from one experiment of five performed are shown. Active p38 MAPK but not active JNK phosphorylates Thr<sup>56</sup> and Ser<sup>87</sup> residues on the Bcl-2 loop region. *B*, rp38 MAPK-GST or recombinant JNK-GST were incubated with rATF-2-GST in the presence of [ $\gamma$ -<sup>32</sup>P]ATP. Samples were run on SDS-PAGE and autoradiographed. rATF-2-GST was phosphorylated by either kinase. *C*, recombinant Bcl-2-GST or its indicated recombinant mutants were incubated in reaction buffer containing ATP in the presence or absence of active rp38 MAPK-GST for 30 min at 30 °C. Reaction mixtures were run on SDS-PAGE, blotted, and stained with anti-phospho-Thr<sup>56</sup>-Bcl-2 Abs or anti-phospho-Ser<sup>87</sup>-Bcl-2 Abs. Reaction mixtures were immunoblotted also with anti-Bcl-2 Abs to ascertain the presence of the recombinant protein in each sample. Results from one experiment of three performed are shown. The anti-phosphosite antibodies (*Ab*) recognize specifically their target phosphoresidues and no other phosphosites within the Bcl-2 sequence.



**FIGURE 4. Immunoblotting analysis of phosphorylated Bcl-2 in CESS cells upon K252A-induced activation of p38 MAPK.** CESS cells were incubated in the presence of 200 nM K252A for 2 h in the presence or absence of 25 mM SB203580, lysed, and immunoblotted with anti-phospho-Ser<sup>87</sup> or anti-phospho-Thr<sup>56</sup>-Bcl-2 antibodies and then with anti-Bcl-2 antibodies, as indicated. Results from one experiment of three performed are shown. p38 MAPK phosphorylates Thr<sup>56</sup> and Ser<sup>87</sup> residues of Bcl-2 upon K252A treatment, a condition known to activate p38 MAPK.

order to demonstrate the relevance of this pro-apoptotic molecular pathway in a cellular system, we obtained embryonic fibroblasts from p38 $\alpha$  wild type mice (p38 $\alpha^{+/+}$  MEF) and knock-out (p38 $\alpha^{-/-}$  MEF) mice (24) and induced their apoptosis by serum withdrawal.

Cell lysates from p38 $\alpha^{+/+}$  MEF or p38 $\alpha^{-/-}$  MEF, cultured in the presence or absence of serum, were collected at different times, and activation of p38 MAPK and phosphorylation of Bcl-2 were revealed by Western blotting techniques with specific antibodies. Fig. 5*A* shows that expression of activated p38

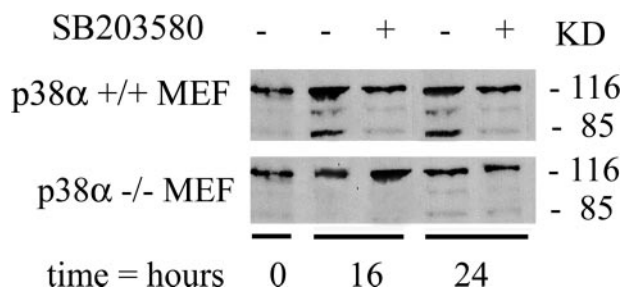


**FIGURE 5. Effect of serum withdrawal on p38 MAPK activation and Bcl-2 phosphorylation in p38 $\alpha^{+/+}$  MEF and p38 $\alpha^{-/-}$  MEF.** *A*,  $8 \times 10^5$  p38 $\alpha^{+/+}$  and p38 $\alpha^{-/-}$  MEF were cultured in the absence of serum for the indicated times, lysed, and immunoblotted with anti-phosphorylated p38 MAPK (*upper gel*) or with anti-total p38 MAPK (*lower gel*). Activated p38 MAPK was evident only in p38 $\alpha^{+/+}$  MEF. *B*,  $8 \times 10^5$  p38 $\alpha^{+/+}$  and p38 $\alpha^{-/-}$  MEF were cultured in the presence or absence of SB203580 p38 MAPK inhibitor for the indicated times, lysed, and immunoblotted with anti-Bcl-2 Abs. Bcl-2 phosphorylation, shown as low migrating 29-kDa band, was induced by serum deprivation only in p38 $\alpha^{+/+}$  MEF and inhibited by specific p38 MAPK inhibitor. Lack of expression of this kinase by gene knock-out inhibits the phosphorylation of Bcl-2.

MAPK started 6 h after serum starvation in p38 $\alpha^{+/+}$  MEF, but not in p38 $\alpha^{-/-}$  MEF. Staining with anti-total p38 MAPK antibodies revealed that the enzyme, obviously never expressed in p38 $\alpha^{-/-}$  MEF, is normally expressed in control p38 $\alpha^{+/+}$  MEF.

p38 $\alpha^{+/+}$  MEF and p38 $\alpha^{-/-}$  MEF were also cultured in the presence or absence of the p38 MAPK inhibitor SB203580 for 12 h. Fig. 5*B* shows the presence of a slowly migrating band of Bcl-2 (29 kDa) in the cell extracts after staining with specific anti-Bcl-2 antibodies, suggesting the occurrence of Bcl-2 phosphorylation. The phenomenon was already evident after 6 h of serum withdrawal only in p38 $\alpha^{+/+}$  MEF and was specifically inhibited by SB203580. In contrast, phosphorylated Bcl-2 protein was not detected in lysates of p38 $\alpha^{-/-}$  MEF. These results indicate that activation of p38 MAPK is responsible for Bcl-2 phosphorylation induced by serum starvation in MEF.

According to the results reported above, in a second series of experiments, we studied whether activated p38 MAPK could induce cytochrome *c* release from mitochondria after serum starvation of MEF. p38 $\alpha^{+/+}$  MEF or p38 $\alpha^{-/-}$  MEF were seeded in Permanox slide chambers and cultured in serum-free conditions. After 6, 9, and 12 h, cells were fixed with paraformaldehyde, permeabilized with Triton X-100, stained with fluorescein isothiocyanate anti-cytochrome *c* and a 4',6-diamidino-2-phenylindole nuclear dye, and analyzed by fluorescence microscope. Cytochrome *c* distribution analysis showed a typical mitochondrial localization in both p38 $\alpha^{+/+}$  and p38 $\alpha^{-/-}$  MEF at time 0. A diffuse cytosolic localization of cytochrome *c* was evident in a small percentage of p38 $\alpha^{+/+}$  MEF ( $18 \pm 2$ ), but not in p38 $\alpha^{-/-}$  MEF. After 9 h, the percentage of p38 $\alpha^{+/+}$  MEF with cytosolic localization of cytochrome *c* further increased ( $50 \pm 3$ ), and morphologic changes of nuclei began to appear, indicating an active apoptotic process. By contrast, the mitochondrial localization of cytochrome *c* was not altered in p38 $\alpha^{-/-}$  MEF. Furthermore, after a 6-h serum starvation,



**FIGURE 6. Effect of p38 MAPK inhibitor on caspase-3-like activation induced by serum withdrawal.** p38 $\alpha$ <sup>+/+</sup> and p38 $\alpha$ <sup>-/-</sup> MEF were cultured in the presence or absence of SB203580 p38 MAPK inhibitor for the indicated times, lysed, and immunoblotted with anti-PARP Abs. PARP cleavage, as a measure of caspase-3-like activation, was detected only in p38 $\alpha$ <sup>+/+</sup> MEF and was inhibited by SB203580. Results from one experiment of three are shown. Caspase-3-like activation was clearly delayed in p38 $\alpha$ <sup>-/-</sup> MEF.

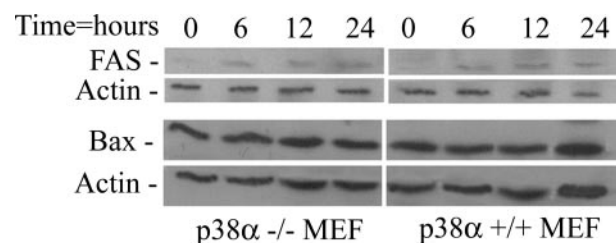
mitochondrial structure was profoundly altered, showing the typical apoptotic organization known as megamitochondria (31) in p38 $\alpha$ <sup>+/+</sup> MEF, but not in p38 $\alpha$ <sup>-/-</sup> MEF (data not shown).

Next, we cultured p38 $\alpha$ <sup>+/+</sup> MEF and p38 $\alpha$ <sup>-/-</sup> MEF under conditions of serum deprivation for different times in the presence or absence of the specific inhibitor SB203580 and studied PARP cleavage as a measure of caspase-3-like enzyme activation. Fig. 6 shows that, after 16 h, PARP cleavage is already present in p38 $\alpha$ <sup>+/+</sup> MEF and is inhibited by SB203580. In contrast, PARP cleavage was not evident at the same time in p38 $\alpha$ <sup>-/-</sup> MEF, thus suggesting that expression and activation of p38 MAPK play a primary role in apoptosis induced by serum starvation.

It was reported that p38 $\alpha$ -deficient fibroblasts are more resistant to apoptosis induced by different stimuli than p38 $\alpha$ -positive cells (32). The reduced apoptosis was correlated with decreased expression of the mitochondrial proapoptotic protein Bax and of the apoptosis-inducing receptor Fas/CD-95 as well as with increasing ERK MAPK activity (32). In order to ascertain whether the reportedly altered expression of apoptotic proteins could take part in the apoptotic patterns observed in our experiments, we cultured p38 $\alpha$ <sup>+/+</sup> MEF and p38 $\alpha$ <sup>-/-</sup> MEF in the absence of serum for different times (6–24 h), and studied Bax and Fas/CD-95 expression by Western blotting analysis. Fig. 7 shows that Bax and Fas/CD-95 expression was not altered, in either p38 $\alpha$ <sup>+/+</sup> MEF or p38 $\alpha$ <sup>-/-</sup> MEF, from 6 to 24 h after serum starvation. These data suggest that in the early phases of serum starvation (6–24 h), cytochrome *c* release from mitochondria is not mediated by increased synthesis of proapoptotic proteins but is probably the consequence of p38 MAPK-mediated Bcl-2 phosphorylation. However, 36 h after serum withdrawal, a slight increase in Fas as well as in Bax protein was detected in p38 $\alpha$ <sup>+/+</sup> MEF (data not shown), suggesting that multiple apoptotic pathways are engaged in the events that take place in this model, following serum deprivation.

## DISCUSSION

One of the most common mechanisms for regulating protein activity is phosphorylation, an event that generally leads to a conformational change of the protein, with an increase or decrease in its activity, largely depending on alterations of the ligand/substrate binding properties. The members of the Bcl-2



**FIGURE 7. Effect of serum withdrawal on Bax and Fas/CD95 production by p38 $\alpha$ <sup>+/+</sup> and p38 $\alpha$ <sup>-/-</sup> MEF.** p38 $\alpha$ <sup>+/+</sup> and p38 $\alpha$ <sup>-/-</sup> MEF were cultured in absence of serum for the indicated times, lysed, and immunoblotted with anti-Fas/CD95 and anti-Bax Abs. Membranes were stripped and stained with anti-actin Abs as protein loading control. Results from one experiment of three performed are shown. Bax and CD95 Fas expression are not increased in p38 $\alpha$ <sup>+/+</sup> and p38 $\alpha$ <sup>-/-</sup> MEF after 24 h of serum withdrawal.

family of proteins usually have a long half-life, and their regulation by alterations of protein expression is limited (33); however, phosphorylation events may modulate their biological activity by triggering or inhibiting the apoptotic pathway without affecting gene expression. Furthermore, Bcl-2 may be the target of different kinases, and multiple conformational changes are expected, depending on the number and location of the phosphorylated residues. Thus, it is not surprising that, at least for Bcl-2, phosphorylation is reported either as increasing or decreasing its biologic activity (6). Defining exactly the kinases involved in Bcl-2 phosphorylation, their target sites within the Bcl-2 sequence, and the functional changes induced by the phosphorylation events can help design both diagnostic and therapeutic strategies to limit or enhance the antiapoptotic activity of Bcl-2 proteins by using specific kinase modulators.

As mentioned above, whether Bcl-2 phosphorylation enhances or decreases the antiapoptotic potential of the protein is still controversial (6). However, in given experimental conditions and under the action of specific kinases, the antiapoptotic function of the protein is clearly decreased (14, 15, 17). In previous papers, we described p38 MAPK as the enzyme responsible for Bcl-2 phosphorylation in memory B lymphocytes upon withdrawal of their survival factor, NGF (13, 14); under these experimental conditions, p38 MAPK was the sole kinase responsible for Bcl-2 phosphorylation. In the present study, we identified, by MALDI-TOF mass spectrometry and immunoblot techniques with specific anti-Bcl-2-phosphopeptides, the two residues Ser<sup>87</sup> and Thr<sup>56</sup> as those phosphorylated by p38 MAPK.

MALDI mass analysis of tryptic Bcl-2 digests, suitable to detect single charged ions at large MW, allowed us to demonstrate that p38 MAPK phosphorylates two residues within the loop region between the  $\alpha$ 1 and  $\alpha$ 2 helices of Bcl-2. Although exact mapping of the phosphorylation site by MS-MS analysis was not successful due to the highly hydrophobic characteristic of the two identified Bcl-2 phosphopeptides, the use of specific anti-Bcl-2 phosphopeptide antibodies, together with Bcl-2 T56A and S87A mutants, allowed us to definitely identify Thr<sup>56</sup> and Ser<sup>87</sup> as Bcl-2 targets of p38 MAPK enzymatic action. These data are consistent with our previous results showing that transfection of B cell lines with the Bcl-2 mutants T56A and S87A makes the cells more resistant to NGF withdrawal-induced apoptosis. In this paper, we clearly demonstrated that activated p38 MAPK is able to induce release of cytochrome *c*

## p38 MAPK and Bcl-2 Phosphorylation

from isolated mitochondria of the Bcl-2-negative MDCK canine cell line only after the addition of recombinant Bcl-2, underscoring the relevance of Bcl-2/p38 MAPK interaction in triggering the apoptotic events. In this experimental system, the T56A/S87A Bcl-2 double mutant, as well as the S87A or T56A Bcl-2 point-mutated recombinant proteins, showed an increased ability to prevent cytochrome *c* release by activated p38 MAPK, as compared with Bcl-2 WT. This evidence suggests that p38 MAPK-induced phosphorylation of the Ser<sup>87</sup> and Thr<sup>56</sup> residues of Bcl-2 may induce conformational changes able to decrease the antiapoptotic potential of the protein.

In a monomeric model of the murine antiapoptotic protein Bcl-2, constructed by comparative modeling using human Bcl-X<sub>L</sub> as a template, it was shown that phosphorylation of Ser<sup>70</sup> may result in a conformational change that aids homo/heterodimer formation by exposing hydrophobic residues in helix 2 important for the formation of dimers (34). Based on these observations, we hypothesize that also phosphorylation of Ser<sup>87</sup> and Thr<sup>56</sup> may inhibit homo/heterodimer formation. Accordingly, some studies have demonstrated that phosphorylated Bcl-2 loses its capacity to heterodimerize with Bax (35, 36). However, the ability of p38 MAPK to induce cytochrome *c* release from isolated mitochondria (*i.e.* in the absence of massive Bax translocation from cytosol) through Bcl-2 phosphorylation suggests that phosphorylation-induced conformational changes of Bcl-2 can also compromise its ion channel-forming properties or its interaction with components of the permeability transition pore. Further studies are needed to clarify this point.

Both in isolated mitochondria and in “*in vitro*” kinase assays, activated JNK, here used in recombinant form, failed to phosphorylate Bcl-2, but not its nuclear substrates. Although in some experimental systems the direct role of JNK in Bcl-2 phosphorylation cannot be denied, since transfection with a dominant negative and/or the use of specific inhibitors abolishes the phenomenon, recent evidence indicates that, at least following paclitaxel treatment, different kinases, such as CDK1, mediate Bcl-2 phosphorylation (20). Consistently, during paclitaxel treatment, lack of synchrony between JNK activation (early event) and Bcl-2 phosphorylation (late event) has been reported (21). Thus, it is conceivable that JNK activation is only the first step in a cascade of events leading to Bcl-2 phosphorylation and that the ability of activated JNK to release cytochrome *c* from isolated mitochondria, reported by some authors (15), is rather related to Bcl-X<sub>L</sub> phosphorylation, as reported by others (37).

In this paper, we also demonstrate the relevance of p38 MAPK in apoptosis induced in a general condition of growth/survival factor deprivation, such as serum starvation (38). Removal of this kinase by gene knock-out strongly delays apoptosis induced by serum deprivation in mouse embryonic fibroblasts, as compared with WT, p38 $\alpha^{+/+}$  MEF. These data are consistent with those reported by Porras *et al.* (32). However, whereas these authors correlate the protection from apoptosis with a decreased synthesis of the mitochondrial proapoptotic protein Bax and the apoptosis-inducing receptor Fas/CD-95 and with increased ERK MAPK activity, we demonstrated that, at least in the early phase of serum deprivation (6–24 h), such an event does not occur. Furthermore, we observed that the

release of cytochrome *c*, occurring a few hours after serum starvation in WT p38 $\alpha^{+/+}$  MEF, is not evident at the same time in p38 $\alpha^{-/-}$  MEF, thus suggesting that Bcl-2 phosphorylation mediated by p38 MAPK might be responsible for cytochrome *c* release from mitochondria, as suggested by *in vitro* experiments, and that both events are crucial at least in the early phases of MEF apoptosis. The observation that both Fas and Bax protein levels increase at later times, probably complementing the initial trigger provided by cytochrome *c* release, indicates that a complex interplay of multiple pathways operated in serum withdrawal conditions. On the whole, these results suggest that Bcl-2 phosphorylation induced by p38 MAPK may be considered a pathway of prominent importance in those acute pathological conditions, such as hypoxia/ischemia, leading to a rapid activation of cell death programs. Consistent with this hypothesis, activation of p38 MAPK was reported in cell death caused by cardiac injury following ischemia-reperfusion *in vivo* (39), and ischemia- and doxorubicin-induced cardiomyocyte apoptosis in culture is specifically attenuated by p38 MAPK inhibitors (40–44).

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**Bcl-2 Phosphorylation by p38 MAPK: IDENTIFICATION OF TARGET SITES  
AND BIOLOGIC CONSEQUENCES**

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