Bcl-2 Undergoes Phosphorylation by c-Jun N-terminal Kinase/ Stress-activated Protein Kinases in the Presence of the Constitutively Active GTP-binding Protein Rac1*

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We have studied the phosphorylation of the Bcl-2 family of proteins by different mitogen-activated protein (MAP) kinases. Purified Bcl-2 was found to be phosphorylated by the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) p54-SAPKβ, and this is specific insofar as the extracellular signal-regulated kinase 1 (ERK1) and p38/RK/CSBP (p38) catalyzed only weak phosphorylation. Bcl-2 undergoes similar phosphorylation in COS-7 when coexpressed together with p54-SAPKβ and the constitutive Rac1 mutant G12V. This is seen by both 32PPO4, labeling and the appearance of five discrete Bcl-2 bands with reduced gel mobility. As anticipated, both intracellular p54-SAPKβ activation and Bcl-2 phosphorylation are blocked by co-transfection with the MAP kinase specific phosphatase MKP3/PYST1. MAP kinase specificity is also seen in COS-7 cells as Bcl-2 undergoes only weak phosphorylation when co-expressed with enzymatically activated ERK1 or p38. Four critical residues undergoing phosphorylation in COS-7 cells were identified by expression of the quadruple Bcl-2 point mutant T56A,S70A,T74A,S87A. Sequencing phosphopeptides derived from tryptic digests of Bcl-2 indicates that purified GST-p54-SAPKβ phosphorylates identical sites in vitro. This is the first report of Bcl-2 phosphorylation by the JNK/SAPK class of MAP kinases and could indicate a key modification allowing control of Bcl-2 function by cell surface receptors, Rho family GTPases, and/or cellular stresses.

Programmed cell death (PCD)§ is an essential process for tissue development and homeostasis as well as for elimination of damaged cells. Failure to control PCD appropriately can lead to diseases involving either inadequate or unwanted cell death (e.g. cancers and neurodegeneration) (1–3). Several intracellular molecules controlling PCD have now been identified. These include Bcl-2, which is a homologue of the Caenorhabditis elegans gene ced-9 and blocks apoptosis under a variety of conditions (3–6). In vertebrates, several Bcl-2 family members have now been identified and include both repressors (e.g. Bcl-2, Bcl-XL, Mc1-1, and A1) and promoters (e.g. Bax, Bcl-XS, Bak, and Bad) of PCD (3, 7). These genes are characterized by their ability to form complex networks of homo- and heterodimers, and their relative abundance probably plays a major role in determining sensitivity to apoptotic signals (3, 8–10). One major unanswered question in relation to control of apoptosis by Bcl-2 family members is of their regulation by extracellular signals or external environment and, importantly, whether receptor-linked transduction pathways target this gene family as a means of determining cell fate.

Emerging data imply that the Bcl-2 gene family are targets for phosphorylation suggesting one potential mechanism of control. For instance, taxol is a chemotherapeutic agent known to promote death, and recent studies in leukemic, prostate, and nasopharyngeal carcinoma cells show that this is accompanied by Bcl-2 phosphorylation (11–13). Bcl-2 has also been reported to be phosphorylated in Jurkat cells (14, 15). Interestingly, Bcl-2 has been reported to associate with Raf-1, which targets this kinase to mitochondrial membranes where the Bcl-2 homologue Bad appears to be a phosphorylation target (16). Together, these observations support the notion that phosphorylation of Bcl-2 family members could be a pivotal mechanism for integrating pro- and anti-apoptotic signals. The molecular identity of protein kinases underlying such phosphorylation is a question of paramount importance to our understanding of PCD.

MAP kinases comprise the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38/RK/CSBP (p38) as three structurally and functionally distinct enzyme classes. Multiple genes and splice variants for each MAP kinase class have also been identified (17–20). The ability of different MAP kinases to phosphorylate Bcl-2 family members has not hitherto been reported. This is surprising given many reports of increased ERK activity by growth and survival factors and moreover, preferential activation of JNK/SAPK by several stimuli and cell stresses known to lead to PCD. In both Jurkat T cells and B104 lymphoma cells, for instance, sustained JNK/SAPK activation by some pro-apoptotic stimuli (γ radiation, UV-C, and anti-IgM) correlates with onset of PCD (21, 22). Consistent with this, overexpression of the kinase ASK-1 or the Fas-binding protein Daax leads to activation of JNK/SAPK as well as enhanced apoptosis in several cell lines (23, 24). Moreover, in PC12 cells, use of mutant kinases indicates that activation of JNK/SAPK together with inhibition of ERK are critical for induction of apoptosis following nerve growth factor with-
JNK/SAPK Phosphorylation of Bcl-2

In this paper we report that JNK/SAPK, and not other classes of MAP kinase tested, is able to phosphorylate Bcl-2, suggesting a role as target substrate in pathways controlling apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction and DNA modifying enzymes were purchased from New England Biolabs Inc. (Beverly, MA) or Life Technologies, Inc., and Tag DNA polymerase was from Perkin-Elmer. [γ-32P]ATP (5000 Ci/mmol), [γ-32P]ATP (1000 Ci/mmol), and [32P]orthophosphate (8500 Ci/mmol) was from DuPont de Nemours International S. A. (Regensdorf, Switzerland). Dulbecco's modified Eagle's cell culture medium was purchased from Life Technologies, Inc. (Beverly, MA), and fetal calf serum was from New England Biolabs Inc. (Beverly, MA) or Life Technologies, Inc., and phenol red-free Dulbecco's modified Eagle's cell culture medium was grown in 6-well plates (35 mm diameter) to 80% confluence and transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. Transfections were performed using the following plasmid concentrations: 0.1 μg of pEE Bcl-2 or pEE Bcl-2 (T56A,S70A,T74A,S87A); 1.0 μg of pcDNA1-HA-p44 ERK1, pMT2T-PA-p54-SAPKβ; or pcDNA3-HA-p38; 1.0 μg of pJX40-HA-Racl (G12V), pJX40-HA-SEK1 (T17N), pJX40-HA-MEK1 (T17N), and 0.05, 0.1, or 1.0 μg of pMT-SM-Myc-MPK3, pMT-SM-Myc-MPK3 (C293S), or pMT-SM-Myc-MPK4 in the combinations indicated in the text. Total plasmid concentrations were maintained constant by supplementing with appropriate empty vectors. Following 6 h of incubation with LipofectAMINE and plasmid DNA, cells were washed and grown for 40 h before lysis and extraction. For acute stimulation of MAP kinase activation, cells were starved by incubation in serum-free medium for 2 h followed by exposure to EGF (10 nM), anisomycin (10 μg/ml), or H2O2 (0.5 mM) at 37 °C for 10–30 min as indicated.

**Cell Culture, Transfection, and Stimulation**—COS-7 cells were grown under 7.5% CO2 in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum and 2 mM glutamine. Cells were grown in 6-well plates (35 mm diameter) to 80% confluence and transfected using LipofectAMINE (Life Technologies, Inc.).

**Cell Lysis and Western Blotting**—Cells were washed twice in 2 ml of ice-cold phosphate-buffered saline and scraped into Eppendorf tubes with 300 μl of buffer TFF (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM EDTA, 10 mM calyculin, and 25 mM β-glycerophosphate). Cells were then homogenized using a sonicator probe at full power for 2 s on ice. For immunodetection of Bcl-2, aliquots of cell lysates (20 μg of protein) were diluted in 10 × Laemmli sample buffer (30), heated at 95 °C for 3 min, and separated by SDS-polyacrylamide gel electrophoresis followed by electrotransfer to nitrocellulose membranes as described (31, 32). Bcl-2 was detected using anti-Bcl-2 antibodies SC 509 or SC 492 and goat anti-mouse or goat anti-rabbit IgG horseradish peroxidase conjugates following stimulation with tumor necrosis factor-α (50 ng/ml) for 20 min at 4 °C. For Bcl-2 immunoprecipitation, supernatant was then mixed by rotary mixing for 2 h at 4 °C with 50 μl of a preformed Bcl-2 immunoprecipitation complex (50 μl of Bcl-2 monoclonal antibody OM-11—925 preincubated with 300 μl of 50% (v/v) protein G-Sepharose and 300 μl of 50% (v/v) protein G-Sepharose beads in 10 mM Tris-HCl, pH 7.5, for 2 h at 4 °C). Beads were then sedimented by centrifugation at 10,000 × g for 3 min and washed four times in 1.0 ml of ice-cold buffer TFF followed by final resuspension in 25 μl of Laemmli sample buffer (30). Immunoprecipitation of HA-tagged MAP kinases was performed with buffer TFF without deoxycholate or SDS using HA-epitope specific monoclonal antibodies HA.11 (HA) and 9E10 (Myc) as described (31, 32).

**Immunoprecipitation and Immune Complex Kinase Assays**—For immunoprecipitation, aliquots (200 μl) of the COS-7 cell lysates were mixed with 800 μl of buffer TFF and rotary mixed for 1 h at 4 °C, after which time they were centrifuged at 100,000 × g for 20 min at 4 °C. For Bcl-2 immunoprecipitation, supernatant was then mixed by rotary mixing for 2 h at 4 °C with 50 μl of a preformed Bcl-2 immunoprecipitation complex (50 μl of Bcl-2 monoclonal antibody OM-11—925 preincubated with 300 μl of 50% (v/v) protein A-Sepharose and 300 μl of 50% (v/v) protein G-Sepharose beads in 10 mM Tris-HCl, pH 7.5, for 2 h at 4 °C). Beads were then sedimented by centrifugation at 10,000 × g for 3 min and washed four times in 1.0 ml of ice-cold buffer TFF followed by final resuspension in 25 μl of Laemmli sample buffer (30). Immunoprecipitation of HA-tagged MAP kinases was performed with buffer TFF without deoxycholate or SDS using HA-epitope specific monoclonal antibodies HA.11 exactly as described (31, 32). For MAP kinase phosphorylation assays, final resuspension was in 50 μl of buffer K (50 mM HEPES, pH 7.4, containing 20 mM MgCl2, 200 μM sodium vanadate, 2 mM dithiothreitol, and 10 μM β-glycerophosphate). Immune complex assays were performed by mixing supernatants containing 5 μM of [γ-32P]ATP (~300,000 dpm/pmol), 10 μM of substrate protein (10 μg of Bax, 10 μg of Bcl-2, 15 μg of MBP, 10 μg of GST-ATF-2, or 10 μg of GST-MAPKAP kinase 2 33) and 30 μl of buffer K followed by incubation for 30 min at 30 °C. Reactions were terminated by adding 15 μl of 10× Laemmli sample buffer (30) and heating for 5 min at 95 °C. Following centrifugation at 10,000 × g for 5 min, supernatants were analyzed by...
RESULTS AND DISCUSSION

To test for in vitro phosphorylation by different MAP kinases we first employed Bcl-2 and Bax proteins purified following expression in E. coli. HA-tagged ERK1, p54-SAPKβ, and p38 were expressed in COS-7 cells and activated by exposure to EGF (10 nm, 10 min), anisomycin (10 μg/ml, 30 min) and hydrogen peroxide (500 μM, 30 min), respectively, as described previously (33). Following immunoprecipitation and incubation with their known substrates MBP, ATF-2, and MAP kinase-activated protein kinase-2 (MK2), all three MAP kinases were confirmed to be enzymatically activated by these stimuli (Fig. IA). Despite this, no phosphorylation of Bax was detectable using any MAP kinase (not shown). In contrast, Bcl-2 phosphorylation was increased by up to 6-fold by incubation with activated p54-SAPKβ (Fig. 1B). Bcl-2 phosphorylation by either ERK1 or p38 MAP kinases was limited to approximately 2-fold under identical conditions (Fig. 1B). Bcl-2 was also phosphorylated 4-fold by immunoprecipitates of activated p46 JNK1 (SAPKγ) (not shown), suggesting selective phosphorylation by the JNK/SAPK class of MAP kinases.

We next tested whether Bcl-2 could be phosphorylated by p54-SAPKβ within the environment of an intact mammalian cell. Human Bcl-2 was expressed in COS-7 cells and found to be immunodetectable as a 26-kDa protein (Fig. 2A). To test for phosphorylation, Bcl-2 was co-expressed with both p54-SAPKβ and the constitutive Rho family G-protein Rac1 (G12V). This GTPase defective mutant has been shown previously to result in moderate activation of JNK/SAPK family members (34, 35), and this is confirmed here with p54-SAPKβ (Fig. 2B). Consistent with in vitro observations using purified protein, Bcl-2 becomes highly phosphorylated under these conditions. This can be readily detected using Western blot analysis as a band shift of immunodetectable Bcl-2 protein (Fig. 2A). Decreased gel mobility of phosphorylated Bcl-2 is consistent with previous reports of a Bcl-2 band shift upon phosphorylation by an unidentified kinase (11, 12). Up to five distinct bands are clearly visible using this technique. The same bands can also be seen.

**Fig. 1.** In vitro phosphorylation of Bcl-2 by activated JNK/SAPK. COS-7 cells were transfected with HA-tagged MAP kinases using 1.0 μg of plasmid and LipofectAMINE. After 40 h of growth and 2 h of serum starvation, cells were incubated in the absence or presence (±) of 10 nM EGF (HA-ERK1), 10 μg/ml anisomycin (HA-SAPKβ), or 0.5 mM H2O2 (HA-p38). MAP kinases were immunoprecipitated using anti-HA epitope-specific monoclonal antibody prebound to protein A-Sepharose beads, and activation state was measured by incubation with MBP, GST-ATF2 (ATF2), GST MAP kinase-activated protein kinase-2 (MK2), or purified recombinant Bcl-2 in the presence of γ-32P-ATP (see "Experimental Procedures"). A, autoradiograph of phosphorylated MBP, ATF-2, and GST MAP kinase-activated protein kinase-2 following separation on a 15% SDS-polyacrylamide gel. Substrate bands were excised and counted by scintillation spectrometry, and the data were used to calculate kinase activity. This is indicated numerically below each lane containing activated MAP kinase (±) as fold stimulation over basal measured in unstimulated control cells (−). B, autoradiograph of phosphorylated Bcl-2 following incubation with the same immunoprecipitants shown in A. Bands were excised and used to calculate Bcl-2 phosphorylation relative to background. This is indicated numerically below each lane.
Although with poorer resolution, upon autoradiographic analysis of Bcl-2 immunoprecipitated from COS-7 cells prelabeled with $[^{32}\text{P}]{\text{H}}_3\text{PO}_4$ (not shown). This phosphorylation appears dependent on enzymatic activation of p54-SAPK$\beta$ because no band shifts are visible when Bcl-2 is expressed either alone with p54-SAPK$\beta$ or with p54-SAPK$\beta$ together with the dominant inhibitory mutant Rac1 (T17N) (Fig. 2, A and B). Limited Bcl-2 phosphorylation observed upon co-expression with Rac G12V alone probably reflects activation of endogenous JNK/SAPK.

Because purified Bcl-2 is phosphorylated most effectively in vitro by JNK/SAPK family members (see above), we set out to test whether similar MAP kinase specificity can be demonstrated within cells. Bcl-2 was coexpressed with combinations of HA-tagged ERK1 as well as constitutively activated Ras (G12V) or dominant inhibitory Ras (T17N) (Fig. 2C). Under conditions leading to ERK1 activation by Ras (G12V) (Fig. 2D), Bcl-2 phosphorylation was barely detectable as indicated by the appearance of only two weak immunodetectable Bcl-2 bands displaying slowed gel mobility (Fig. 2C). This effect was not seen when Bcl-2 was expressed alone with either Ras (G12V) or ERK1, or in combination with ERK1 and inactive Ras (T17N) (Fig. 2C). We have also performed similar experiments where Bcl-2 is coexpressed with p38 MAP kinase alone or together with Rac1 (G12V) or Rac1 (T17N). In these experiments (not shown), despite p38 MAP kinase activation, no phosphorylation was seen over and above that noted when Bcl-2 was expressed alone with Rac1 (G12V) (not shown).

To confirm that Bcl-2 phosphorylation in the presence of p54-SAPK$\beta$ and Rac1 (G12V) is a reflection of intracellular MAP kinase activation, we assessed Bcl-2 phosphorylation in COS-7 cells coexpressing the MAP kinase phosphatases MKP3/PYST1 and MKP4 (31, 32, 36). In addition to Bcl-2, p54-SAPK$\beta$, and Rac1 (G12V), cells were also transfected with increasing quantities of plasmid encoding MKP3/PYST1 or MKP4 (0.05–1.0 $\mu$g of plasmid/well). This resulted in a dose-dependent increase in levels of immunodetectable MKP3/PYST and MKP4 protein (see Ref. 32), and this was accompanied by both a graded inhibition of p54-SAPK$\beta$ enzymatic activity and a complete disappearance of four of the five additional Bcl-2 bands detected by Western analysis (not shown). The fifth Bcl-2 band showing reduced gel mobility was also weakened considerably by the highest level of MKP3 (not shown).

The three-dimensional structure of the Bcl-2 homologue Bcl-X$_r$ reveals an unstructured 60-residue flexible loop connecting two $\alpha$-helices localized immediately N-terminal of the conserved BH3 homology domain (37). Structural modeling of Bcl-2 predicts a similar unstructured loop, and within this region we identified two serines (residues 70 and 87) and two threonines (residues 56 and 74), each preceding prolines that we reasoned represent potential sites for phosphorylation by p54-SAPK$\beta$. In support of this, expression of the Bcl-2 mutant T56A,S70A,T74A,S87A in COS-7 cells abolished four of the five additional Bcl-2 bands normally observed upon coexpression with p54-SAPK$\beta$ and Rac1 (G12V) (Fig. 3). This experiment indicates that one or more of these residues represent essential target sites for phosphorylation following activation of p54-SAPK$\beta$ within COS-7 cells. The site of the modification underlying the single remaining low mobility band is unknown. To test whether p54-SAPK$\beta$ is able to modify these sites directly, we next phosphorylated Bcl-2 in vitro in the presence of $[^{32}\text{P}]{\text{P}}\text{ATP}$ using purified active GST-p54-SAPK$\beta$. Following
trypsin digestion of phosphorylated Bcl-2, HPLC separation resolved 13 peptides (Fig. 4A), although only two peaks of 33P-radioactivity were detected in the entire elution profile (Fig. 4B). These peaks correspond to peptides 6 and 10, which were sequenced by Edman degradation and found to be amino acids 27–68 and 69–98 of Bcl-2, respectively (Fig. 4C). Together, these two peptides include all four residues undergoing phosphorylation following p54-SAPK β activation in intact cells.

While these studies were in progress a report appeared describing Bcl-2 expression in the immature B cell line WEHI-231 exposed to anti-IgM (38). In these cells full-length Bcl-2 was ineffective at suppressing cell death and strikingly also undergoes extensive phosphorylation visible as multiple bands with retarded gel mobility. A Bcl-2 deletion mutant lacking the predicted flexible loop region was not phosphorylated and remarkably was able to block apoptosis in these cells. Although the kinase responsible for phosphorylating Bcl-2 was not identified in this report (46), our results suggest that JNK/SAPK family members may be responsible. Indeed, in another B cell line (B104) cross-linking mIgM triggers sustained activation of JNK/SAPK, and this is accompanied by cellular apoptosis (32). Together with our data, these observations are consistent with a model whereby pro-apoptotic signals such as mIgM cross-linking activate members of the JNK/SAPK MAP kinase family, which then disable Bcl-2 through phosphorylation within its flexible loop region. Such Bcl-2 inactivation would be expected to reinforce pro-apoptotic processes such as mitochondrial membrane depolarization, generation of reactive oxygen intermediates, and activation of cysteine proteases of the caspase family.

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