Nerve Growth Factor Inhibits Apoptosis in Memory B Lymphocytes via Inactivation of p38 MAPK, Prevention of Bcl-2 Phosphorylation, and Cytochrome c Release*

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Maria Torcia‡§§, Giovanna De Chiara‡, Lucia Nencioni‡, Serena Ammendola‡, Danilo Labardi‡, Maria Lucibello‡, Paolo Rosini‡, Lionel N. J. L. Marlier‡¶¶, Paolo Bonini‡¶¶, Persio Dello Sbarba‡¶¶, Anna Teresa Palamara‡§§, Nicola Zambrano¶¶, Tommaso Russo¶¶, Enrico Garaci‡, and Federico Cozzolino¶¶

From the Departments of Experimental Medicine and Internal Medicine, University of Rome “Tor Vergata,” Via di Tor Vergata 135, Rome I-00133, Italy, the Department of Clinical Physiopathology and Institute of General Pathology, University of Florence, Viale Pieraccini 6, Florence I-50139, Italy, the Department of Cellular and Molecular Biology and Pathology “L. Califano” and Department of Biochemistry and Medical Biotechnologies, University of Naples “Federico II,” Via Pansini 5, Naples I-80131, Italy, and Institute of Neurobiology and Molecular Medicine, National Research Council, Via del Fosso del Cavaliere 100, Rome I-00133, Italy

Survival of memory B lymphocytes is tightly linked to the integrity of the Bcl-2 protein and is regulated by a nerve growth factor (NGF) autocrine circuit. In factor-starved memory B cells, the addition of exogenous NGF promptly induced p38 mitogen-activated protein kinase (MAPK), but not c-Jun N-terminal kinase (JNK), dephosphorylation. Conversely, withdrawal of endogenous NGF was followed by p38 MAPK activation and translocation onto mitochondria, whereby it combined with and phosphorylated Bcl-2, as assessed by co-immunoprecipitation and kinase assays in vitro and in vivo. Mitochondria isolated from human memory B cells, then exposed to recombinant p38 MAPK, released cytochrome c, as did mitochondria from Bcl-2-negative MDCK cells loaded with recombinant Bcl-2. Apoptosis induced by NGF neutralization could be blocked by the specific p38 MAPK inhibitor SB203580 or by Bcl-2 mutations in Ser-87 or Thr-56. These data demonstrate that the molecular mechanisms underlying the survival factor function of NGF critically rely upon the continuous inactivation of p38 MAPK, a Bcl-2-modifying enzyme.

Apoptosis is a highly structured and ordered process that in multicellular organisms eliminates superfluous, harmful, and metabolically perturbed cells (1, 2). It can be triggered by specialized surface receptor molecules, such as CD95 or taxol, or by stimuli as diverse as withdrawal of growth factors, exposure to cell “poisons” (such as staurosporine or taxol), corticosteroids, viral infections, or even hypoxia. All of these signals converge on the mitochondrial crossroads, whereby critical events, such as cytochrome c efflux and formation of the apoptosome, take place under the control exerted by proteins of the Bcl-2 family (3–5).

It is established that the proapoptotic members of the family, such as Bax, Bad, or Bag, form pores in the outer mitochondrial membrane triggering cytochrome c release and that a subset of the family, the so-called “BH3-only” proteins (Bid, Bim, Noxa, Harakiri, etc.), enrolled in various cell types and metabolic conditions, facilitate their oligomerization and membrane insertion, initiating or augmenting their function (6). On the other hand, the proposed mechanisms accounting for the function of the anti-apoptotic Bcl-2 proteins are diverse and somewhat less clear. In fact, Bcl-2 and Bcl-XL can either directly combine with and inhibit the proapoptotic members or indirectly modulate their activity by interfering with the mitochondrial membrane events they cause (4, 5). In parallel, a number of studies have demonstrated that Bcl-2 and Bcl-XL can bind to the mitochondrial proteins adenine nucleotide translocator (7) and voltage-dependent anion channel (8, 9), suggesting that their regulatory function can operate through the influence on the gating properties of the latter, probably by keeping it in the open configuration, thus allowing the exchange of metabolites between cytosol and mitochondria (10). If so, structural modifications affecting Bcl-2 or Bcl-XL might modify or even reverse their antiapoptotic potential (11). In this connection, phosphorylation of Bcl-2 has been described to profoundly alter its function, but contrasting reports have shown either loss (12–17) or gain (18–22) of antiapoptotic properties after enzymatic modification.

In multicellular organisms, the fate of a given cell is under the social control exerted by the surrounding elements, which deliver life or death signals. A class of molecular inputs that keep alive the cell are the survival factors, whose surface receptors transmit signals that inhibit the pathways leading to cell death. Nerve growth factor (NGF) is the first and perhaps most representative protein of this class (23). Since NGF withdrawal in factor-dependent cells typically affects Bcl-2 protein (17, 24–27), we wanted to define the molecular events linking...
NGF surface receptors with Bcl-2 in memory B lymphocytes, a cell type that depends on a functional NGF autocite circuit for survival (24). In the present paper, we describe that, upon factor withdrawal, p38 mitogen-activated protein kinase (MAPK) in its active form rapidly translocates onto mitochondria, specifically interacts with Bcl-2, and phosphorylates it, an event that is followed by cytochrome c release from the organelle and by apoptotic cell death. Transfection experiments with Bcl-2 mutants bearing substitutions of Ser-87 or Thr-56 residues within the loop region showed increased resistance to apoptosis induced by NGF withdrawal. The observation that p38 MAPK is promptly dephosphorylated after NGF administration to factor-starved cells indicates that the cytokine modulates the enzyme activation to maintain survival of NGF-dependent cells.

**EXPERIMENTAL PROCEDURES**

Reagents—Neutralizing rat anti-human NGF mAbs (clone d11) were kindly donated by Dr. A. Cattaneo (International School for Advanced Studies, Trieste, Italy), and always used at 10 μg/ml. Rabbit anti-human p38 MAPK, anti-human JNK, anti-human phosphorylated JNK, anti-human actin, anti-human PARP, and goat and mouse anti-human Bcl-2 Abs were purchased by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-human phosphorylated p38 MAPK was purchased by New England Biolabs. Anti-human hsp60 was purchased by Stressgen. Human recombinant active p38 MAPK-GST, JNK-GST, and Bcl-2-GST was purchased by Upstate Biotechnology, Inc. (Lake Placid, NY). HrATF2-GST was purchased by Santa Cruz Biotechnology. Human recombinant NGF was a generous gift of Dr. G. Ferrari (Fidia, Abano, Italy). The p38 MAPK inhibitor SB203580, the inactive SB202474, the PD98059 MEK1/2 inhibitor, and the Trk-A inhibitor K252a were purchased by Calbiochem. Anisomycin was purchased by Sigma.

Human recombinant Bcl-2-His, (1–218) was expressed with a C-terminal His6 tag using pQE60 vector (Quiagen) in M15 cells (Escherichia coli). Recombinant protein was purified by resuspending a 1-liter cell cultures in 50 mM phosphate buffer, pH 8, with 300 mM NaCl, 10 mM imidazole. The cell suspension was incubated with 1 mg/ml lysozyme for 30 min at 4 °C, sonicated to reduce viscosity, and centrifuged at 10,000 × g for 30 min. The supernatant was applied to a nickel-activated Hi-Trap™ chelating column (Amersham Pharmacia Biotech) and purified with an FPLC apparatus (Amersham Pharmacia), using a wash step with 50 mM phosphate buffer, 300 mM NaCl, 30 mM imidazole, pH 8.0, until the A280 reached <0.01. Proteins were eluted with 250 mM imidazole, desalted against 10 mM Hepes, 150 mM NaCl, 20 mM MgCl2 as running buffer. The purity of each fraction was assessed by staining aliquots on 10% SDS-PAGE. Gels were dried and autoradiographed on x-ray films.

**Phosphorylation Assays**—For in vitro kinase reactions, p38 MAPK coupled to Sepharose beads or 2 μg/ml rabbit anti-p38 MAPK Abs coupled to Sepharose beads or 2 μg/ml rabbit anti-JNK or with 2 μg/ml control rabbit IgG. The efficacy of immunoprecipitation was checked by Western blot analysis with anti-P38 MAPK Ab or anti-JNK Ab. Immune complexes were incubated with 0.25 μg of human recombinant Bcl-2-GST protein or Bcl-2-His, or 0.25 μg of rATF-2-GST and 3 μc of [γ-32P]ATP (specific activity 3,000 Ci/mmol, Amersham Pharmacia Biotech), in the presence of 25 μM SB203580, or Me_SO as control, in 50 μl of kinase buffer (25 mM Hepes, pH 7.4, with 25 mM β-glycerophosphate, 2 mM Na_2HPO_4, 0.1 mM NaVO_3, 1 mM phenylmethylsulfonyl fluoride, 0.25 mM dithiothreitol, 0.25 mM Na_2EDTA, 1 mM Na_2HPO_4, and 1 μM leupeptin) at 30 °C for 30 min. After 30 min at room temperature, samples were suspended in 2-ME sample buffer, run in SDS-PAGE, dried, and autoradiographed on Kodak XAR films for 12 h at −20 °C. A kinase assay was performed in the same experimental conditions by using 0.25 μg of recombinant active p38 MAPK GST or JNK-GST and 0.25 μg of Bcl-2-His.

To assess the activity of recombinant proteins, 0.25 μg of active rp38 MAPK or rJNK-GST were incubated, with or without 25 μM SB203580, with 0.25 μg of rATF-2-GST in kinase buffer for 30 min in the presence of 3 μc of [γ-32P]ATP. Samples were run on SDS-PAGE and autoradiographed.

**Cytokine c Release**—Mitochondrial and cytosolic (S100) fractions from human tonsils, and sIgD Abs, as described (24). Cells were cultured in RPMI 1640

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**Bcl-2 Phosphorylation by p38 MAPK**

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**Surface Plasmon Resonance Analysis**—Surface plasmon resonance analysis was performed using a BIACORE instrument with CMS sensor chips and an amine coupling kit (BIACORE AB). For immobilization of proteins, the sensor chip was equilibrated with HBS buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, pH 7.4, plus 0.005% β-d-mannose) at 5 μl/min and then activated by injecting 35 μl of 0.2 N ethyl-N-3-dimethylaminopropylcarbodiimide, 0.05 M N-hydroxysuccinimide. 35 μl of kinase or control protein at −20 μg/ml in 10 mM sodium acetate, pH 4.5, were then injected. The binding curves were evaluated with the BIAevaluation software.
were prepared by resuspending cells (3 × 10^6) in 0.8 ml of mitochondrial isolation buffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 2 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride protease inhibitor, 2 μg/ml leupeptin, 10 μg/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, 1,500 g spin. The supernatant was spun at 10,000 g for 30 min at 4 °C, and the pellet containing the mitochondrial fraction was suspended in modified mitochondrial isolation buffer with 75 mM MgCl2, containing active p38 MAPK or active JNK (0.25 μg), 200 μM ATP, and 25 mM β-glycerophosphate. After incubation at 30 °C for 30 min, the mitochondrial mixture reactions were then centrifuged at 4,000 × g for 10 min, and supernatant and pellet proteins were separated on SDS-PAGE, followed by transfer to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech). The blot was probed with anti-human cytochrome c peptide mAb (7H8:2C12, Pharmigen), diluted at 1:500. A secondary probe (1:5,000 dilution) with horseradish peroxidase-labeled antibodies (Amersham Pharmacia Biotech) was detected by ECL (Amersham Pharmacia Biotech).

MDCK cells, detached by trypsin-EDTA, were washed twice in phosphate-buffered saline and suspended in 8 ml of mitochondrial isolation buffer. Mitochondrial fractions were obtained as above and 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 Bcl-2-His6 or transformed with empty plasmid, for 30 min on ice and 15 min at 22 °C. The resulting deleted cDNA was purified, digested with BglII and EcoRI restriction endonucleases, and ligated in the MCS of pIRE2-EGFP. All constructs were verified by sequencing analysis.

**RESULTS**

NGF Controls p38 MAPK Activation—In the NGF-responsive cell line PC12, deprivation of NGF is followed by rapid phosphorylation of two members of the MAPK family, p38 and JNK (28–31). In order to assess these events also in memory B lymphocytes, lysates from cells, initially starved (i.e. cultured at low cellular density (12 h at 2 × 10^5/ml) to dilute the autocrine factor) and then treated or not with exogenous NGF, were analyzed in Western blots with anti-phosphorylated p38 MAPK or with anti-phosphorylated JNK Abs, which revealed p38 MAPK activation in NGF-starved cells, rapidly declining after NGF addition (Fig. 1A); in contrast, equal amounts of phosphorylated JNK were observed under the same conditions (Fig. 1A). These results suggested that in memory B cells p38 MAPK was specifically involved in the NGF signaling, the difference between neural cells and memory B cells probably residing in the expression of p38 MAPK-specific, but not JNK-specific, phosphatase(s) in the latter cells, as described in other systems (32).

Next, to characterize the molecular pathway comprising activated p38 MAPK and antagonized by NGF, we checked whether neutralization of endogenous NGF signaling with specific Abs (or with Trk-A inhibitor K252a, not shown) caused enzyme hyperphosphorylation, which indeed occurred at early times, as expected on the basis of similar results by others (28), while the total amount of protein increased at later times (Fig. 1B). In contrast, JNK was not hyperphosphorylated in the same experimental conditions (Fig. 1B).

Mitochondrial Localization of Activated p38 MAPK—p38 MAPK is a serine-threonine kinase, whose known molecular targets are nuclear, such as ATF-2 or GADD153, or cytosolic, such as hsp27, proteins (33). In the inactive state, its subcellular location is the cytosol, from where it is translocated to the nucleus, following metabolic activation (34). We next determined by immunofluorescence, using Abs specific for the activated form of p38 MAPK, the amount of memory B cells that scored positive upon NGF neutralization, detecting 73 ± 5% of brightly staining cells, versus 7 ± 2% in control cells, in three independent experiments. During these studies we observed, besides the nuclear staining, a spotted pattern of cytoplasmic staining (within a diffuse cytosolic context), that suggested an additional localization of the activated enzyme. Thus, to assess in detail the physical translocation events of activated p38 MAPK, confocal laser-scanning microscopy experiments were performed on memory B cells treated with anti-NGF or control IgG and with Mitotracker (a rhodamine-like mitochondrial dye) and then fixed and stained with p38 MAPK-specific Abs. Fig. 4A shows that, after neutralization of endogenous NGF in memory B cells, a definite mitochondrial localization of p38 MAPK from its cytosolic, diffuse location was evident, a localization of the enzyme that was so far undetected. Staining with specific

**Construction of Bcl-2 Mutants**—The full-length human-bcl-2 sequence was amplified by polymerase chain reaction from lysates of freshly isolated human lymphocytes and cloned as a BglII/EcoRI fragment in pIRE2-EGFP vector (CLONTECH), using the following primers: 5'-TTAGATCTTCGCGGAGCGCTAGA-3' (forward) and 5'-CCGAATTCTCACTTGTGGCTCAGATAGG-3' (reverse), yielding the wild type (WT) construct. To replace Thr-56, Ser-70, Thr-74, and Ser-87 besides the nuclear staining, a spotted pattern of cytoplasmic staining (within a diffuse cytosolic context), that suggested an additional localization of the activated enzyme. Thus, to assess in detail the physical translocation events of activated p38 MAPK, confocal laser-scanning microscopy experiments were performed on memory B cells treated with anti-NGF or control IgG and with Mitotracker (a rhodamine-like mitochondrial dye) and then fixed and stained with p38 MAPK-specific Abs. Fig. 4A shows that, after neutralization of endogenous NGF in memory B cells, a definite mitochondrial localization of p38 MAPK from its cytosolic, diffuse location was evident, a localization of the enzyme that was so far undetected. Staining with specific

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with anti-human phosphorylated JNK or anti-human phosphorylated blotted with 1/H9262. A superimposable amounts of protein in each lane. Results of one treated as above, were lysed and immunoblotted with anti-human phosphorylated JNK Abs and then with anti-human JNK. sIgD IgG, lysed, and immunoblotted with rabbit anti-human phosphorylated p38 MAPK Abs and then with anti-human p38 MAPK. sIgD B cells were treated for the indicated times with 10 μg/ml anisomycin for 2 h, lysed, and immunoblotted with anti-human phosphorylated p38 MAPK Abs and then with anti-human p38 MAPK. sIgD B cells, treated as above, were lysed and immunoblotted with anti-human phosphorylated JNKAbs and then with anti-human JNK. Results of one experiment out of three performed are shown. Gel loading control, performed by staining both membranes with anti-actin Abs, revealed superimposable amounts of protein in each lane. C, sIgD B cells were stimulated with 10μg/ml anisomycin for 2 h, lysed, and immunoblotted with anti-human phosphorylated JNK or anti-human phosphorylated p38 MAPK. Abs revealed that essentially only the activated enzyme translocated to mitochondria (Fig. 2A).

Next, to confirm the above results, a biochemical analysis was performed on mitochondrial, nuclear, and cytosolic fractions from cells treated or not with anti-NGF Abs. Fig. 2B shows that p38 MAPK was detected in cytosol and nuclei of cells exposed to control Abs and that the enzyme also accumulated in mitochondria of cells treated with anti-NGF Abs. Interestingly, when the same filters were stained with Abs against the active form of p38 MAPK, specific bands were evident in the mitochondrial and nuclear fractions and completely undetectable in the cytosolic fraction (Fig. 2C). Thus, neutralization of NGF interaction with surface receptors was reflected into activation of a serine/threonine-kinase, whose mitochondrial localization opened the question regarding the function exerted by the enzyme in that compartment.

p38 MAPK Binds Bcl-2—Several proteins, including enzymes, bind Bcl-2 (4) (e.g., the serine-kinase Raf-1, which localizes in mitochondria of cells co-transfected with Bcl-2 and Raf-1 genes) (35, 36). We therefore wanted to ascertain whether p38 MAPK was able to combine with Bcl-2. Mitochondria were purified from 108 memory B cells treated with anti-NGF or control Abs, and either Bcl-2 or p38 MAPK was immunoprecipitated, run in SDS-PAGE, and blotted onto filters developed with anti-p38 MAPK or anti-Bcl-2 Abs, respectively. Fig. 3A shows that p38 MAPK co-purified with Bcl-2, particularly in cells exposed to anti-NGF Abs. The presence of a variable number of apoptosing cells at the end of separation procedures accounts for the baseline amount of p38 MAPK on mitochondria; a densitometric analysis of gels from replicate experiments revealed a 62 ± 18% increase of p38 MAPK co-immunoprecipitated by anti-Bcl-2 Abs from lysates of anti-NGF-treated cells (not shown). Fig. 3B shows the reverse analysis, performed by immunoprecipitating p38 MAPK and revealing Bcl-2 co-purification. To ascertain whether the two proteins interacted directly, without the participation of adapter or scaffold molecules, we performed the same experiments using recombinant proteins only, which showed that indeed this was the case (Fig. 3, C and D). In all of the above experiments, specificity of immunoprecipitation was assessed by staining the filters with the same Abs used in the first step of the procedure, revealing that indeed the Abs had recognized the nominal antigen.

Although Bcl-2 interacts with several molecular partners in vivo (4), a quantitative assessment of its physical interaction with a given protein is not available. In this connection, and to gather further evidence of the ability of p38 MAPK and Bcl-2 to combine, we set up experiments employing a biosensor-based analysis, in which interaction of proteins, expressed in recombinant form, was monitored as a function of time (37). Fig. 3E shows that Bcl-2 efficiently combined with p38 MAPK-GST coupled to the solid phase (520 RU); in contrast, when JNK-GST was tested under the same conditions, lower degrees of interaction were detected (240 RU). The latter way of looking at the interaction of different kinases with Bcl-2, namely in terms of pure physical interaction, further confirms that p38 MAPK combines with Bcl-2.

p38 MAPK Phosphorylates Bcl-2—The notion that Bcl-2 undergoes phosphorylation on several serine and threonine residues within the loop between BH4 and BH3 domains (38), which modifies its antiapoptotic potential (14, 17), raised the question of whether the serine/threonine kinase p38 MAPK could catalyze that reaction. To assess whether Bcl-2 phosphorylation by p38 MAPK occurred in cells deprived of NGF, 32P]ATP. Fig. 4A shows that phosphorylated Bcl-2 was recovered from anti-NGF-treated cells but not from cells treated with NGF and that only the addition of SB203580 inhibited phosphorylation. Again, specificity and efficiency of Bcl-2 immunoprecipitation were assessed in parallel experiments run under the same experimental conditions (Fig. 4A). These experiments were performed in the presence of the phosphatase inhibitor okadaic acid; however, superimposable results were obtained also in its absence (data not shown; see Ref. 17). A similar approach with JNK, based on the reports stating that also this enzyme can phosphorylate Bcl-2 (14, 40–44), could not be followed, because the described JNK inhibitors were unavailable to us. Thus, to confirm the above findings in a cell-free system, p38 MAPK and JNK were purified from memory B cells exposed to anti-NGF Abs and tested on recombinant Bcl-2 and ATF-2 proteins in the presence of [32P]ATP. Fig. 4B shows that indeed the latter proteins became phosphorylated after 30 min when exposed to p38 MAPK and that the reaction was clearly modulated by SB203580. In contrast, no Bcl-2 and little ATF-2 phosphorylation was evident upon exposure to purified JNK, consistent with the above described evidence that NGF withdrawal failed to activate JNK in the memory B cell system.
Finally, both p38 MAPK and JNK were obtained in recombinant form and tested in a Bcl-2 phosphorylation assay in the presence of \(^{32}\)P-ATP, which again demonstrated that, under the experimental conditions used, p38 MAPK, but not JNK, was specifically active on Bcl-2 (Fig. 4C). In contrast, Fig. 4D shows that both p38 MAPK and JNK preparations were active on the common ATF-2 substrate and that SB203580 was able to inhibit p38 MAPK activity only. Consistent results were obtained when Bcl-2 phosphorylation by p38 MAPK (both in recombinant form) was assessed in a BIAcore analysis, in that...
anti-phosphoserine Abs, flowed through the reaction chamber after removal of the enzyme, specifically recognized serine residues(s) on immobilized Bcl-2 (not shown).2

On the whole, this set of experiments showed that Bcl-2 phosphorylation by activated p38 MAPK is a crucial event in the metabolic cascade modulated by NGF in memory B cells.

Activated p38 MAPK Induces Cytochrome c Release from Mitochondria and Apoptosis—The phosphorylation process induces significant conformational changes, which may confer diverse functional properties to proteins (45), and in fact phosphorylation of different members of the Bcl-2 family was shown to increase or abolish their function (35, 46, 47). Bcl-2 phosphorylation was shown, in contrasting reports, to enhance or in-  

other companion proteins, experiments were performed on mitochondria isolated from MDCK, a canine cell line that does not express Bcl-2 (48). Recombinant Bcl-2 was added to mitochondria and allowed to insert in the membrane, and then recombinant activated p38 MAPK or JNK were added, and the release of cytochrome c was assessed after 2 h. Fig. 5B shows that only p38 MAPK, and not JNK, elicited cytochrome c release and that again SB203580 was able to specifically inhibit the phenomenon (by \(-68\%\), as assessed by densitometric analysis of gels from the three experiments performed). In particular, the addition of either enzyme to mitochondria not previously exposed to Bcl-2 failed to cause release of cytochrome c, indicating that they did not recognize any endogenous protein involved in the translocation process.

The above experiments strongly suggested that Bcl-2 phosphorylation by p38 MAPK was causally related to cytochrome c release. Consistently, it is remarkable that cleavage of PARP (Fig. 5C) and the ensuing apoptosis of memory B cells (Fig. 5D), both induced by NGF neutralization, could be prevented by SB203580.

As a whole, these findings point to Bcl-2 phosphorylation by p38 MAPK as the key event leading memory B cells to apoptosis upon NGF deprivation.

Mutant Bcl-2 Proteins Inhibit Apoptotic Death after NGF Withdrawal—We next wanted to extend the above observations, by assessing which serine and/or threonine residue of Bcl-2 protein was involved in p38 MAPK-induced phosphorylation, by employing selected Bcl-2 mutants. Since resting cells can be transfected with difficulty if at all, we used CESS cells,

2 G. De Chiara, M. E. Marcacci, F. Pichiorri, M. Lucibello, A. L. Rosa, P. Rosini, A. T. Palamara, E. Garaci, M. Torcia, and F. Cozzolino, manuscript in preparation.

Fig. 3. Bcl-2 and p38 MAPK interaction. A, mitochondrial fractions, obtained from 106 slgD \(-\) B cells, treated with 10 µg/ml anti-NGF Abs or control IgG for 4 h, were lysed and immunoprecipitated (IP) with anti-human Bcl-2 Abs (lanes 1 and 2) or with control mouse Ig (lanes 3 and 4), run on SDS-PAGE (control IgG-treated cells (lanes 1 and 3) and anti-NGF-treated cells (lanes 2 and 4)), blotted, and stained with anti-human p38 MAPK Abs. The same nitrocellulose filter was stripped and restained with anti-human Bcl-2 Abs. Results of one experiment out of four performed are shown. B, mitochondrial fractions of 106 slgD \(-\) B cells, treated as in A, were immunoprecipitated with anti-human p38 MAPK Abs (lanes 1 and 2) or rabbit Ig (lanes 3 and 4) and stained with anti-human Bcl-2 Abs (control IgG-treated cells (lanes 1 and 3) and anti-NGF-treated cells (lanes 2 and 4)). The same nitrocellulose filter was stripped and restained with anti-human p38 MAPK Abs. The results of one experiment out of three performed are shown. C, a mixture of rBcl-2-His6 (250 ng) and active rp38 MAPK-GST (250 ng) was immunoprecipitated with anti-Bcl-2 (lane 1) or control Abs (lane 2). The immune complexes were stained with anti-p38 MAPK Abs. Lane 3, rp38 MAPK (internal standard). The same nitrocellulose filter was stripped and restained with anti-human p38 MAPK Abs. Results of one experiment out of three performed are shown. D, a mixture of rBcl-2-His6 (250 ng) and active rp38 MAPK-GST (250 ng) was immunoprecipitated with anti-p38 MAPK (lane 1) or control Abs (lane 2). The immune complexes were stained with anti-human Bcl-2 Abs. Lane 3, rBcl-2-His6 (internal standard). The same nitrocellulose filter was stripped and restained with anti-human p38 MAPK Abs. Results of one experiment out of three performed are shown. E, overlay plot of sensorgrams obtained with Bcl-2-His6 over chips containing immobilized active p38 MAPK-GST, active JNK-GST, or GST. The bindings recorded were 520 RU for active p38 MAPK-GST, 240 RU for active JNK-GST, and 90 RU for GST.
coding for green fluorescent protein (GFP) as reporter. CESS cells were transfected with the above constructs or with a void plasmid, and apoptosis (revealed by cytofluorimetric analysis of annexin-V staining) was recorded after exposure to anti-NGF Abs. Although a significant level of protection from apoptosis was achieved in cells transfected with WT plasmid, as compared with native CESS cells or to mock-transfected cells, the \( \Delta \)-loop mutant presented strong resistance to NGF withdrawal (Fig. 6). When point mutants were tested, we observed that T56A and S70A mutants showed levels of resistance to apoptosis comparable with those of \( \Delta \)-loop mutant, while T74A and S70A mutants did not (Fig. 6); superimposable results were obtained by inducing apoptosis with K252a compound (not shown). The latter findings strongly indicate that phosphorylation of Thr-56 and/or Ser-87 is causally linked to apoptosis induced by withdrawal of NGF in sIgD\(^{-}\) B cells.

**DISCUSSION**

NGF is a classical survival factor, essential for a large number of cell types, including neurons, keratinocytes, and memory B lymphocytes (24–27). In all of these cells, discontinuation of the NGF signal invariably causes apoptotic cell death that critically involves mitochondria with alterations of proteins of the Bcl-2 family, as occurs in different systems after the removal of other survival factors (49). How withdrawal of such factors, particularly of NGF, reverberates onto mitochondria is not completely defined. Our observations delineate a pathway that relays the extracellular signal of NGF or its absence with the maintenance or alteration of Bcl-2 integrity and may therefore be useful for understanding the vertical connections between cell surface and the mitochondrial phase of apoptosis.

We observed that, upon NGF signaling in memory B cells, p38 MAPK was dephosphorylated, while the addition of neutralizing anti-NGF Abs caused its phosphorylation and translocation to mitochondria. This alternative localization of the activated enzyme, so far undescribed, may be related to the phosphorylation status of paxillin, a focal adhesion protein involved in linking actin filaments to the plasma membrane (50), is analogous to the reported translocation of JNK to the same compartment (51), and appears to be dictated by its affinity for Bcl-2, as revealed by the co-localization experiments, the co-immunoprecipitation studies, and the biosensor analysis. Translocation of p38 MAPK to the mitochondrial compartment was also functionally meaningful, as suggested by the ample evidence that the enzyme, in native or recombinant form, can phosphorylate Bcl-2, its activity being specifically counteracted by the inhibitor SB203580 or by the deletion of the Bcl-2 loop region (17). Following Bcl-2 phosphorylation, cytochrome c outpouring, activation of caspase-3-like enzymes (as shown by PARP cleavage), externalization of phosphatidylserine, and DNA degradation occurred in cells treated with anti-NGF Abs, events that could be prevented by SB203580 or by mutations of selected residues of Bcl-2 protein. A final piece of evidence comes from the recent observation made in our laboratory that Bcl-2 phosphorylation does not occur in fibroblasts (28) as described forosphatidylyserine, and DNA degradation occurred in cells treated with anti-NGF Abs, events that could be prevented by SB203580 or by mutations of selected residues of Bcl-2 protein. A final piece of evidence comes from the recent observation made in our laboratory that Bcl-2 phosphorylation does not occur in fibroblasts (28) as described for other cell types that rely solely on Bcl-2 for survival, as further discussed below.

It is believed that the antiapoptotic properties of Bcl-2 and Bcl-X\(_L\) rely on the ability of native molecules to bind a number of proapoptotic members of the family, such as Bad, Bim, Harakiri, Bik, and, possibly, also Bax (6), preventing or inhib-
mitigating their functions. Moreover, Bcl-2 and Bcl-X	extsubscript{L} interact with proteins making up the permeability transition pore complex, particularly VDAC and ANT (7–10, 53), and cooperate in maintaining these molecules in a physiological state. However, when the BH4 domain is removed (11, 54), their properties may switch toward promotion of apoptosis (55). Our data suggest that even more limited modifications, such as phosphorylation, can alter Bcl-2 function, leading to a proapoptotic molecule. In fact, Bcl-2 phosphorylation may act either disrupting the intermolecular cooperation (12, 15, 53) or rendering the protein susceptible to proteolytic enzymes (56). While a number of reports are consistent with our contention (12–14, 16, 17), others reach the opposite conclusion (18–20), suggesting that Bcl-2 has to be phosphorylated in order to act as an antiapoptotic factor. Bcl-2 protein has several residues potentially targeted by serine/threonine kinases in the loop region and linked to apoptosis, as reported by others (14, 58, 57). Our observation that single substitutions of Bcl-2 threonine 56 or serine 87 with alanine caused reduced sensitivity to NGF neutralization strongly indicates that these residues, once targeted by p38 MAPK, trigger cell death. This finding, taken together with the notion that a phosphorylated serine 70 is needed to ensure cell survival after etoposide treatment (18), suggests that a precise combination of activated and deactivated residues dictates the shaping of Bcl-2 folding that governs its gating functions. Deciphering the combination, a task currently under way in our laboratory, should solve the inconsistencies regarding phosphorylation of Bcl-2 protein and its function in the apoptotic process.

We observed that JNK, another member of the MAPK/stress-activated protein kinase family, was not deactivated upon NGF signaling. This finding is in partial accordance with the findings of Kummer et al. (28), who had shown that the enzymatic activities of both JNK and p38 MAPK diminished after administration of NGF to PC12 cells. This difference might be explained by the absence, at this stage of lymphoid maturation, of the enzymatic machinery needed to dephosphorylate JNK upon NGF addition, as occurs to p38 MAPK, since specific phosphatases are involved in the inactivation of the two kinases (32). However, it is also possible that the two members of the MAPK family, JNK and p38, preferentially interact with Bcl-X	extsubscript{L} and Bcl-2, respectively, as suggested by the recent demonstration that JNK specifically phosphorylates Thr-47 and Thr-115 residues in Bel-X, protein (51); if so, since memory B cells do not express Bcl-X, protein (58), the absence of the natural mitochondrial target of JNK would render dispensable the specific cytosolic phosphatase instead needed in PC12 cells that enzymatically targets JNK upon NGF addition. As for Bcl-2, consistent with the hypothesis of preferential interactions of kinases, although JNK was reported to bind and phosphorylate Bcl-2 in cell transfection assays (14, 41), we observed a limited degree of JNK/Bcl-2 interaction in a biosensor analysis. Moreover, in our system,
normal memory B cells, reflecting naturally occurring molecular events, and it is remarkable that the alternative localization of activated p38 MAPK on mitochondria can be observed shortly after a simple exposure of normal cells to neutralizing anti-NGF Abs. On the whole, delineating the vertical connections between a cell surface signal and mitochondria, where the post-translational modifications of Bcl-2 affecting its anti-apoptotic properties occur, the present data provide information that may be helpful in designing pharmacological strategies to influence the survival of memory B cells and probably of other different cell types.

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