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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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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 vivo* and *in vitro*. 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 TNFR, 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-X_L 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 parellel, a number of studies have demonstrated that Bcl-2 and Bcl-X_L 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-X_L 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)[1] 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

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[1] The abbreviations used are: NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; Ab, antibody; mAb, monoclonal antibody; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; MDCK, Madin-Darby canine kidney; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; RU, resonance units; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; GFP, green fluorescent protein; EGFP, enhanced GFP; PE, phycoerythrin; PARP, poly(ADP-ribose) polymerase; NTR, neurotrophin receptor.

NGF surface receptors with Bcl-2 in memory B lymphocytes, a cell type that depends on a functional NGF autocrine 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 αD11) 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 were 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 PD098059 MEK1/2 inhibitor, and the Trk-A inhibitor K252a were purchased by Calbiochem. Anisomycin was purchased by Sigma.

Human recombinant Bcl-2-His_6-(1–218) was expressed with a C-terminal His_6 tag using pQE60 vector (Quiagen) in M15 cells (*Escherichia coli*). Recombinant protein was purified by resuspending 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 A_{280} reached <0.01. Proteins were eluted with 250 mM imidazole, desalted against 10 mM Hepes, 150 mM NaCl, pH 7.4, and stored at −20 °C.

Cell Isolation and Culture—Resting B lymphocytes were obtained from human tonsils, and $sIgD^-$ subpopulation were purified by panning procedures using plastic Petri dishes coated with rabbit anti-human IgD antibodies, as described (24). Cells were cultured in RPMI 1640 medium supplemented with 1% Nutridoma for immunoprecipitation and Western blot analysis.

CESS and MDCK cell lines were purchased by ATCC (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum.

Phosphorylation Analysis of p38 MAPK and JNK—$sIgD^-$ B cells were cultured overnight at 2 × 10^5 cells/ml, washed, and incubated at 10^7 cells/ml with human recombinant NGF (100 ng/ml) or with 10 μg/ml anti-NGF Abs or rat IgG. At different time intervals, cells were washed, lysed in sample buffer, run on SDS-PAGE, blotted onto nitrocellulose filters, and stained with 0.1 μg/ml rabbit anti-human phosphorylated p38 MAPK or rabbit anti-human JNK. The reaction was visualized by ECL detection system as recommended by the manufacturer (Amersham Pharmacia Biotech). Membranes were stripped at 56 °C and stained with 0.1 μg/ml rabbit anti-human p38 MAPK or anti-human JNK.

Immunofluorescence Analysis—For immunofluorescence analysis, $sIgD^-$ cells, treated with 10 μg/ml anti-NGF Abs or control Ig for 4 h, were incubated with 25 nM Mitotracker (Molecular Probes, Inc., Eugene, OR) for 30 min at 37 °C and then attached on poly-L-lysine-sensitized slides. The cells were fixed with 2% paraformaldheyde, permeabilized with 0.1% Triton X-100 in PBS, and, after saturation of slides with 5% low fat dry milk (Bio-Rad) in phosphate buffer, stained with 0.1 μg/ml rabbit anti-human p38 MAPK, anti-human phosphorylated p38 MAPK, or preimmune rabbit IgG, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. The slides were analyzed by a confocal laser-scanning microscope.

Purification of Nuclear, Mitochondrial, and Cytosolic Fraction—10^8 cells were suspended in 5 mM Tris, pH 7.4, with 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, pH 8.0, containing 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.7 μg/ml pepstatin for 30 min on ice, Dounce-homogenized, and centrifuged at 750 × *g* at 4 °C to obtain the nuclear fraction. The supernatant was further centrifuged at 10,000 × *g* for 30 min at 4 °C to obtain the mitochondrial fraction (pellett) and the cytosolic fraction (supernatant). For Western blot analysis, the nuclear and mitochondrial fractions were directly lysed in sample buffer, while the cytosolic fraction was vacuum-concentrated and subsequently suspended in sample buffer. The purity of each fraction was assessed by staining aliquots with Abs to hsp60 for mitochondria, to actin for cytosol, and to PARP for nuclei.

Co-immunoprecipitation Studies—For co-immunoprecipitation studies, mitochondrial fraction from 10^8 $sIgD^-$ cells, treated with 10 μg/ml anti-NGF antibodies or control IgG, were lysed in 10 mM Hepes, 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% Nonidet P-40, pH 7.5, and immunoprecipitated with 1 μg/ml rabbit anti-p38 MAPK Abs, anti-Bcl-2 mAbs, or 1 μg/ml polyclonal rabbit IgG or mouse IgG, followed by protein A-Sepharose or protein G-Sepharose, respectively. The same approach was used with recombinant proteins; a mixture of 200 ng of rBcl-2-His_6 and 200 ng of active rp38 MAPK-GST was immunoprecipitated with 1 μg/ml anti-Bcl-2, or with 1 μg/ml rabbit anti-p38 MAPK Abs or control Abs followed by protein G-Sepharose or protein A-Sepharose. The immune complexes were washed, run on 10% SDS-PAGE, and blotted onto nitrocellulose filter. 100 ng of human recombinant Bcl-2-His or hrp38 MAPK-GST were used as internal standard.

Surface Plasmon Resonance Analysis—Surface plasmon resonance analysis was performed using a BIACORE instrument with CM5 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% p20 surfactant) at 5 μl/min and then activated by injecting 35 μl of 0.2 M *N*-ethyl-*N*′-3-diethylaminopropylcarbodiimide, 0.05 M *N*-hydroxysuccinimide. 35 μl of kinases or control protein at ~20 μg/ml in 10 mM sodium acetate, pH 4.5, were then injected. Excess *N*-hydroxysuccinimide-esters on the surface were deactivated with 35 μl of 1 M ethanoldiamine-hydrochloride (pH 8.5), and noncovalently bound dimers were washed out with 10 μl of regeneration buffer (4 M Gu-HCl buffered with 50 mM phosphate buffer, pH 6.8). Binding experiments were performed with 10 mM Hepes, 150 mM NaCl, 20 mM $MgCl_2$ as running buffer.

Phosphorylation Assays—For *in vivo* metabolic labeling, 10^7 $sIgD^-$ cells were incubated with 0.5 mCi of [^{32}P]orthophosphate for 4 h in the presence of 1 μM okadaic acid or 10 μl of ethanol vehicle control, in phosphate-free medium, in the presence or absence of anti-NGF Abs, 25 μM SB203580, SB202474, PD098059, or 100 ng/ml NGF. Cells were then lysed as described, immunoprecipitated with anti-Bcl-2 Abs as reported above, and separated on 10% SDS-PAGE. Gels were dried and autoradiographed on Eastman Kodak Co. XAR films for 72 h at −20 °C.

Kinase Assay—10^8 $sIgD^-$ cells, treated with rat anti-NGF mAb for 12 h, were lysed in 20 mM Tris, pH 7.4, containing 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 2 mM NaPP, 1 mM $NaVO_3$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin. 400 μg of proteins were immunoprecipitated in duplicate with 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_6 or 0.25 μg of rATF-2-GST and 3 μCi of [^{32}P]ATP (specific activity 3,000 Ci/mmol; Amersham Pharmacia Biotech), in the presence of 25 μM SB203580, or Me_2SO as control, in 30 μl of kinase buffer (25 mM Hepes, pH 7.4, with 25 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM $NaVO_3$). 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 or inactive p38 MAPK-GST or JNK-GST and 0.25 μg of rBcl-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 μCi of [γ-^{32}P]ATP. Samples were run on SDS-PAGE and autoradiographed.

Cytochrome c Release—Mitochondrial and cytosolic (S100) fractions

were prepared by resuspending cells (3×10^7) in 0.8 ml of mitochondrial isolation buffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 2 mM $MgCl_2$, 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, $750 \times g$ spin. The supernatant was spun at $10,000 \times g$ for 30 min at 4 °C, and the pellet containing the mitochondrial fraction was suspended in modified mitochondrial isolation buffer with 75 mM $MgCl_2$, 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 $4,000 \times g$ 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-His_6 or transformed with empty plasmid, obtained by lysing the cells with 20 mM Hepes, pH 7.4, 30 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1% CHAPS. 0.25 μg of active p38 MAPK or JNK, with or without 25 μM SB203580, was then added, and the phosphorylation reaction was performed in the presence of 200 μM ATP (Amersham Pharmacia Biotech) for 30 min at 30 °C. Mitochondrial mixture reactions were then centrifuged at $4,000 \times g$ for 10 min, and then supernatant and pellet proteins were separated on SDS-PAGE, blotted on polyvinylidene difluoride membranes, and stained with anti-human cytochrome *c* mAbs.

Determination of PARP Cleavage, Annexin-V Staining, DNA Fragmentation, and Cell Viability—$sIgD^-$ B cell subpopulations were cultured at 5×10^6/ml, with 10 μg/ml anti-NGF mAb or control rat IgG, in the presence or absence of 25 μM SB203580 for 12 h, lysed with 0.1% Triton X-100 in PBS, run on SDS-PAGE, blotted onto nitrocellulose filters, and stained with 1:1,000 anti-PARP mAbs, followed by peroxidase-conjugated anti-mouse Abs. Reaction was visualized by ECL.

For annexin-V staining, the cells, cultured as above with anti-NGF antibodies in the presence or absence of SB203580, were washed once in PBS, incubated with 5 μg/ml PE-annexin-V (Alexis) in binding buffer (0.01 M Hepes, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$) for 15 min at room temperature. After washing, the cells were analyzed by cytofluorimetry.

For DNA fragmentation analysis, $sIgD^-$, cultured at 5×10^6/ml, with 10 μg/ml anti-NGF mAb or control rat IgG, in the presence or absence of 25 μM SB203580 for 12 h, were diluted 1:1.5 with 5 mM Tris, 20 mM EDTA, and 0.5% (v/v) Triton X-100, pH 8.0, and allowed to lyse for 15 min on ice before centrifugation for 20 min at $27,000 \times g$ to separate intact chromatin (pellet) from DNA fragments (supernatant). Pellets were resuspended in 5 ml of a buffer containing 10 mM Tris and 1 mM EDTA, pH 8.0, and pellet and supernatant samples were assayed for DNA content, using the diphenylamine reagent (1.5% diphenylamine in acetic acid plus 10% acetaldehyde) for 16 h at 30 °C. The optical density at 600 nm was measured for each sample. Percentage of DNA fragmentation was calculated as reported (24). Cell viability was evaluated by trypan blue dye exclusion.

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 *Bgl*II/*Eco*RI fragment in pIRES2-EGFP vector (CLONTECH), using the following primers 5′-TTAGATCTATGGCGCACGCTGGGAGAAC-3′ (forward) and 5′-CCGAATTCTCACTTGTGGCTCAGATAGG-3′ (reverse), yielding the wild type (WT) construct. To replace Thr-56, Ser-70, Thr-74, and Ser-87 with Ala, mutagenesis reactions using a QuikChange™ site-directed mutagenesis kit (Stratagene) were performed. The Δ-loop plasmid, containing the *bcl-2* deletion mutant (Δ40–91 amino acids), was generated by two-step recombinant polymerase chain reaction using WT as template and two oligonucleotide pairs: 5′ - CCACGGGCCCGGCGCCCACATCTCCCGC-3′ with 5′-GCAGAGCTGGTTTAGTGAACCGTCA-3′ and 5′-GGGCCCGTGGTCCACCTGGCCCTCCGCCAA-′ with 5′-TATTCCAAGCGGCTTCGGCCAGTAA-3′.

The two polymerase chain reaction products were mixed in the same polymerase chain reaction tube and reamplified with terminal primers. The resulting deleted cDNA was purified, digested with *Bgl*II and *Eco*RI restriction endonucleases, and ligated in the MCS of pIRES2-EGFP. All constructs were verified by sequencing analysis.

Cell Culture, Transient Transfections, and Cytofluorimetric Analysis—Transient transfections of CESS cells were obtained by LipofectAMINE (Life Technologies, Inc.). 2×10^6 cells were seeded into six-well plates and incubated for 5 h in serum-free medium with 5 μg of test plasmid and lipids, according to the manufacturer's instructions. After 72 h, cells were washed and analyzed by using the FL-1 detector of a FACScan flow cytometer (Becton Dickinson). For each sample, 10^4 cells were analyzed at a rate of 300 cells/s. The percentage of transfected cells was calculated after subtracting the nonspecific fluorescence of nontransfected cells and resulted in ≥30%. Apoptotic stimuli (10 μg/ml of anti-NGF Abs for 12 h or 200 nM K252a for 6 h) were added 72 h after transfection, and GFP^+ transfected cells undergoing apoptosis were detected by PE-annexin-V staining and cytofluorimetric analysis with both FL-1 and FL-2 detectors.

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. 1*A*); in contrast, equal amounts of phosphorylated JNK were observed under the same conditions (Fig. 1*A*). 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. 1*B*). In contrast, JNK was not hyperphosphorylated in the same experimental conditions (Fig. 1*B*).

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. 2*A* shows that, after neutralization of endogenous NGF in memory B cells, a definite mitochondrial focusing of p38 MAPK from its cytosolic, diffuse location was evident, a localization of the enzyme that was so far undetected. Staining with specific

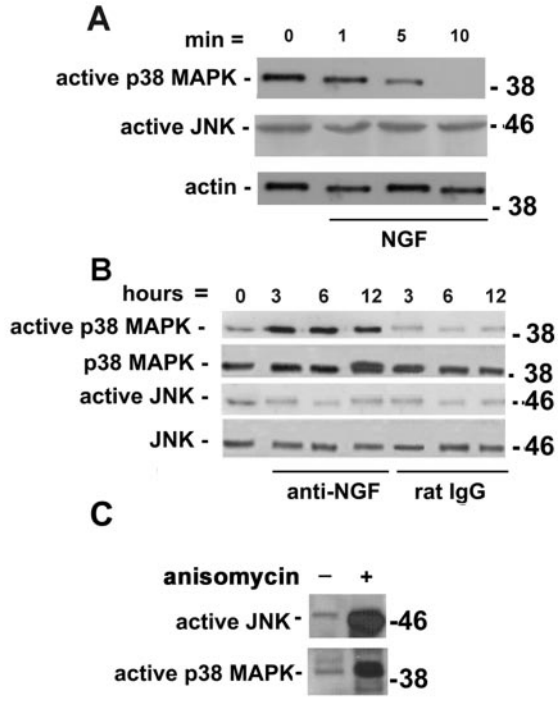

FIG. 1. **NGF induces dephosphorylation of p38 MAPK, but not of JNK.** *A*, cell lysates from sIgD$^-$ memory B cells, cultured with 100 ng/ml human recombinant NGF for the indicated times, were immunoblotted with 1 μg/ml rabbit anti-human phosphorylated p38 MAPK or rabbit anti-human phosphorylated JNK Abs. Restaining of membranes with anti-actin Abs is also shown. Results of one representative experiment out of three performed are shown. *B*, sIgD$^-$ B cells were treated for the indicated times with 10 μg/ml rat anti-NGF mAb or control rat IgG, lysed, and immunoblotted with rabbit 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 JNK Abs 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$^-$ cells were stimulated with 10 μg/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. 2*A*).

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. 2*B* 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. 2*C*). 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 10^8 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. 3*A* shows that p38 MAPK co-purified with Bcl-2, particularly in cells exposed to anti-NGF Abs. The presence of a variable number of apoptosizing 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. 3*B* 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 adaptor 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. 3*E* 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, $^{32}PO_4$ autoradiography experiments were set up by immunoprecipitating Bcl-2 from cells treated with either NGF or anti-NGF Abs, in the presence or absence of SB203580 (a pyridyl-imidazole inhibitor of the enzyme (39)), of SB202474 (an inactive pyridyl-imidazole compound (14)), and of PD098059 (a specific MEK1/2 inhibitor (14)) as controls. Fig. 4*A* 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. 4*A*). 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 [^{32}P]ATP. Fig. 4*B* 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.

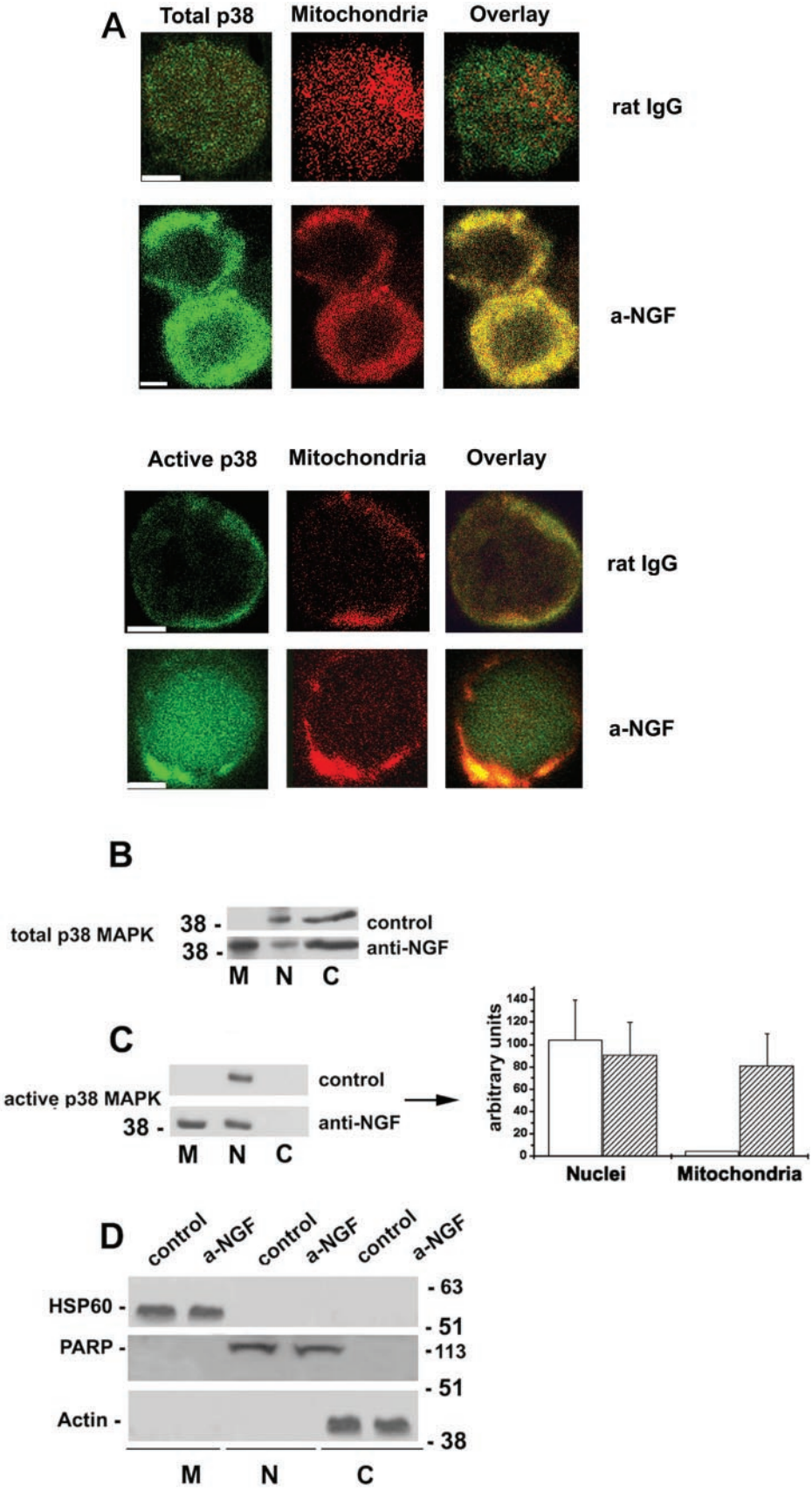

FIG. 2. **Neutralization of endogenous NGF induces phosphorylation and mitochondrial translocation of p38 MAPK.** *A*, immunofluorescence analysis of sIgD$^-$ cells treated with 10 μg/ml anti-NGF Abs or control rat IgG for 4 h. Cells were incubated with Mitotracker and stained with rabbit anti-human p38 MAPK, rabbit anti-human phosphorylated p38 MAPK, or control rabbit IgG, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. The slides were analyzed by a confocal laser-scanning microscope. Mitochondrial localization of p38 MAPK, defined by yellow spots indicating overlap of fluorescein and rhodamine, was absent in samples of cells treated with rat IgG. Staining of cells with preimmune rabbit IgG did not show detectable signal using a fluorescein filter (not shown). *Bar*, 2 μm. *B* and *C*, mitochondrial (*M*), nuclear (*N*), or cytosolic (*C*) fractions of sIgD$^-$ B cells, treated with 10 μg/ml anti-NGF Abs or control IgG for 4 h, were blotted and stained with anti-human p38 MAPK (*B*) or with anti-human phosphorylated p38 MAPK Abs (*C*). All of the cellular fractions derived from anti-NGF-treated cells contained p38 MAPK. In the same cells, the phosphorylated form of the enzyme localized on mitochondria and was undetectable in the cytosol. Densitometric analysis (mean ± S.D.) of active p38 MAPK in nuclei and mitochondria from rat IgG-treated cells (*open bar*) or anti-NGF-treated cells (*hatched bar*) from five different experiments are shown in the graph. Arbitrary units are pixels/0.1 cm^2. *D*, cytosolic, nuclear, and mitochondrial preparations from cells treated with anti-NGF or control IgG were blotted and stained with anti-hsp60, anti-human PARP, and anti-actin Abs to assess the purity of subcellular fractions. For all of the panels, results from one experiment out of five performed are shown.

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. 4*C*). In contrast, Fig. 4*D* 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

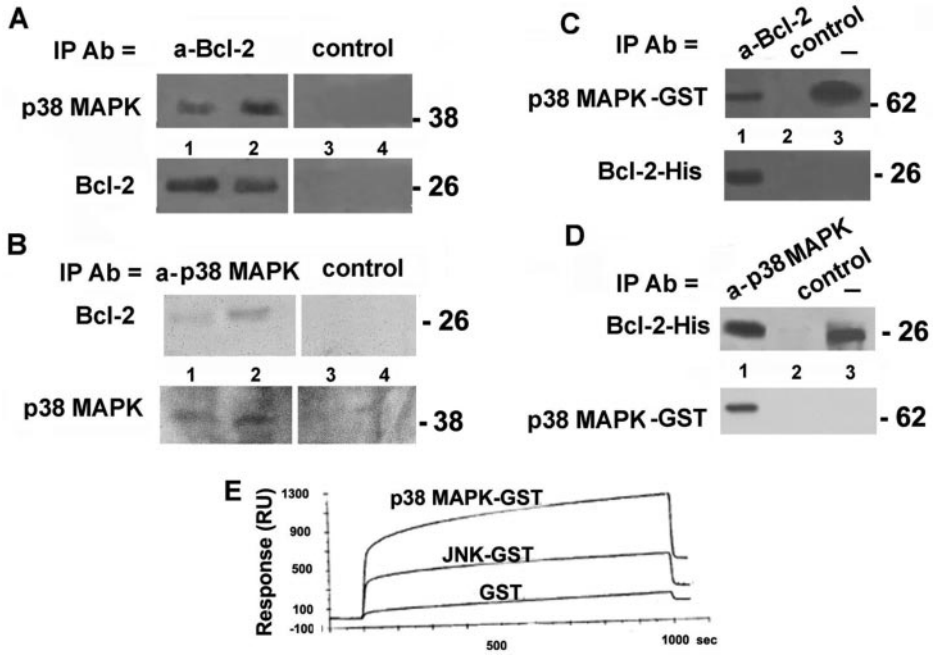

FIG. 3. **Bcl-2 and p38 MAPK interaction.** *A*, mitochondrial fractions, obtained from 10^8 sIgD$^-$ B cells, treated with 10 μg/ml anti-NGF Abs or control IgG for 4 h, were lysed and immunoprecipitated (IP) with anti-human Bcl-2 Abs (*lanes 1* and *2*) or with control mouse Ig (*lanes 3* and *4*), 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 10^8 sIgD$^-$ 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 four performed are shown. *C*, a mixture of rBcl-2-His$_6$ (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 Bcl-2 Abs. Results of one experiment out of three performed are shown. *D*, a mixture of rBcl-2-His$_6$ (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-His$_6$ (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-His$_6$ 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.

anti-phosphoserine Abs, flowed through the reaction chamber after removal of the enzyme, specifically recognized serine residue(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 inhibit its antiapoptotic potential (12, 13, 15–22), in particular to perturb specific functions, such as the control of cytochrome *c* efflux from mitochondria (14). In our system, Bcl-2 phosphorylation was most likely related to the onset of apoptosis. To test this hypothesis, mitochondria were isolated and incubated for 2 h with active p38 MAPK, and then the supernatants were analyzed for the presence of cytochrome *c*. Fig. 5*A* shows that the active form of p38 MAPK induced a dramatic release of cytochrome *c*, which was specifically inhibited by SB203580. When equal amounts of active JNK were tested, no cytochrome *c* release was detected (Fig. 5*A*).

Next, to ascertain that cytochrome *c* release was uniquely related to Bcl-2 phosphorylation, and not to modifications of 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. 5*B* 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 ± 3%, 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 relase 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. 5*C*) and the ensuing apoptosis of memory B cells (Fig. 5*D*), 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. Marcocci, F. Pichiorri, M. Lucibello, A. L. Rosa, P. Rosini, A. T. Palamara, E. Garaci, M. Torcia, and F. Cozzolino, manuscript in preparation.

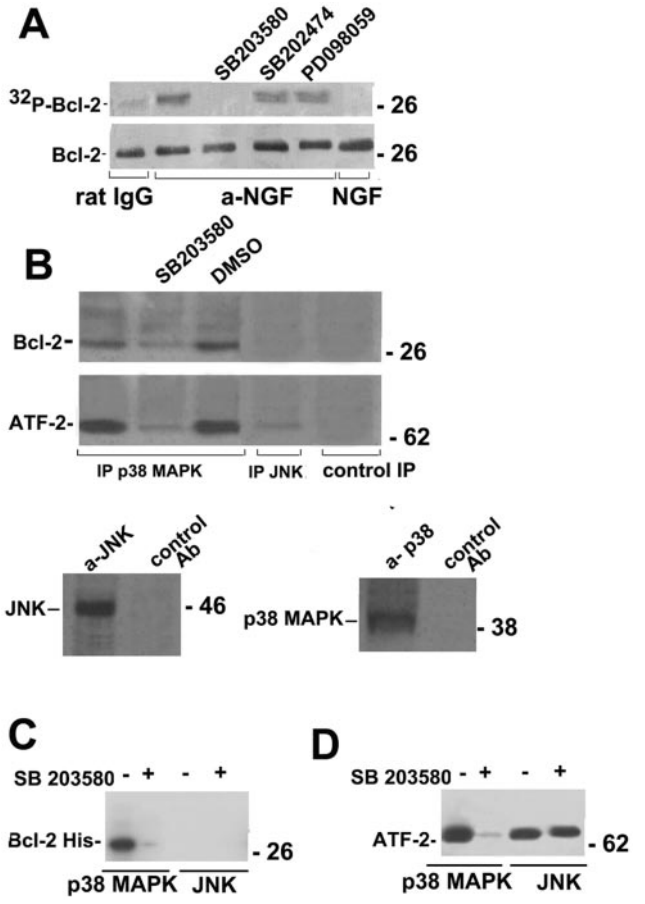

FIG. 4. **Bcl-2 is phosphorylated by p38 MAPK.** *A*, $^{32}PO_4$ metabolic labeling of sIgD$^-$ B cells treated for 4 h with 10 μg/ml control Ig or anti-NGF Abs, with anti-NGF Abs plus 25 μM SB203580, 25 μM SB202474, 25 μM PD098059, or with 200 ng of NGF. Cell lysates were immunoprecipitated with anti-Bcl-2 Abs and run on 10% SDS-PAGE. Duplicate gels were dried and autoradiographed (*upper panel*) or blotted onto nitrocellulose filters and stained with anti-Bcl-2 Abs (*lower panel*). Results from one experiment out of six performed are shown. *B*, *in vitro* kinase assay of immunopurified p38 MAPK or immunopurified JNK from lysates of anti-NGF-treated IgD$^-$ B cells. Immunopurified p38 MAPK, JNK, or control IgG-immunoprecipitated proteins were incubated, in the presence of [γ-^{32}P]ATP, with 250 ng of rBcl-2-His$_6$, rBcl-2-His^6 plus 25 μM SB203580, or rBcl-2-His^6 plus Me_2SO (*DMSO*) as control vehicle. The immunopurified proteins were incubated, in the same experimental conditions, with rATF-2-GST (250 ng). The reactions were stopped after 30 min, and the samples were run on SDS-PAGE, dried, and autoradiographed. Immunopurified p38 MAPK phosphorylates Bcl-2 and ATF-2-GST, while immunopurified JNK does not. Results from one experiment out of three performed are shown. The efficacy of immunoprecipitation was checked by running duplicate immunoprecipitation (*IP*) samples in separate gels and by staining the membranes with anti-human p38 MAPK or anti-human JNK. *C*, active rp38 MAPK-GST or active rJNK-GST were incubated with rBcl-2-His$_6$ (250 ng) in the presence of [γ-^{32}P]ATP. Samples were run on SDS-PAGE and autoradiographed. rBcl-2 was phosphorylated by rp38 MAPK-GST, and the reaction was specifically inhibited by SB203580. Results from one experiment out of three performed are shown. *D*, active rp38 MAPK-GST or rJNK-GST were incubated, in the presence or absence of 25 μM SB203580, with rATF-2-GST (250 ng) in the presence of [γ-^{32}P]ATP. Samples were run on SDS-PAGE and autoradiographed. rATF-2-GST was phosphorylated by either kinase, but SB203580 specifically inhibited rp38 MAPK-GST only. Results from one experiment out of three performed are shown.

which bear a sIgG$^+$ phenotype, produce NGF, and express NGF receptors, thus presenting biochemical features similar to those of memory B cells (17). We cloned WT Bcl-2 and a series of mutants, in which either the coding sequence for the entire loop region of the molecule was deleted (Δ-loop) or Thr-56, Ser-70, Thr-74, or Ser-87 was replaced by Ala in plasmids 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 Δ-loop mutant presented strong resistance to NGF withdrawal (Fig. 6). When point mutants were tested, we observed that T56A and S87A mutants showed levels of resistance to apoptosis comparable with those of Δ-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 from p38 MAPK$^{-/-}$ embryos (52) under serum withdrawal conditions leading to Bcl-2 phosphorylation in control p38 MAPK$^{+/+}$ fibroblasts.[2] On this basis, we suggest that a major biochemical link between NGF receptors and the mitochondrial compartment is p38 MAPK, whose function is therefore critical for apoptosis of memory B cells and, possibly, of the 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-

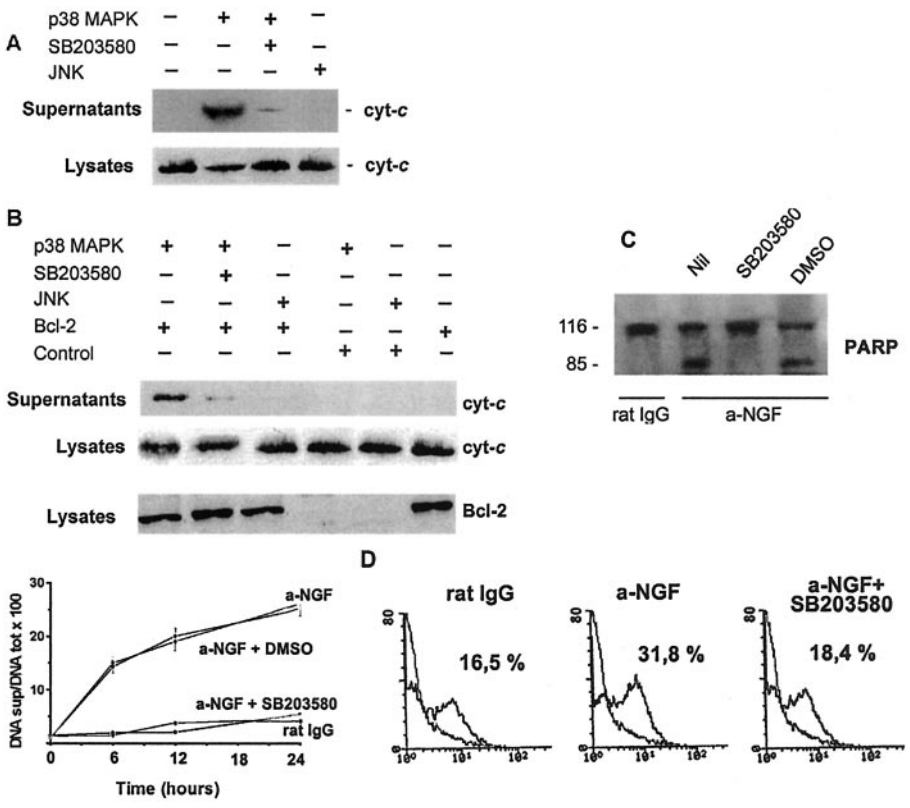

FIG. 5. **p38 MAPK induces release of cytochrome *c* from mitochondria and SB203580 prevents apoptosis.** *A*, mitochondria isolated from sIgD$^-$ B cells were incubated for 2 h with 250 ng of active rp38 MAPK, with active rp38 MAPK in the presence of 25 μM SB203580, or with 250 ng of active rJNK. Reaction supernatants and mitochondrial lysates were immunoblotted with anti-cytochrome *c* Abs. Results from one experiment out of three performed are shown. *B*, mitochondria isolated from MDCK cells were incubated with lysates of *E. coli* cells expressing Bcl-2-His$_6$ or transformed with empty vectors, in the presence of ATP and active rp38 MAPK, active rp38 MAPK plus SB 203580, or active rJNK. Supernatants of reaction mixtures and mitochondrial lysates were run on SDS-PAGE, blotted, and stained with anti-cytochrome *c* Abs. The *lower gel* shows the mitochondrial extracts immunoblotted with anti-Bcl-2 Abs to ascertain that the recombinant protein had inserted into the organelle membranes. Results from one experiment out of three performed are shown. *C*, cell lysates from sIgD$^-$ B cells, incubated with 10 μg/ml rat IgG, anti-NGF Abs, anti-NGF Abs plus 25 μM SB203580, or with Me_2SO (*DMSO*) as control vehicle, were immunoblotted with anti-PARP Abs. PARP cleavage, induced by anti-NGF Abs, was inhibited by SB203580. Results from one experiment out of three performed are shown. *D*, sIgD$^-$ B cells were cultured with anti-NGF Abs or control IgG, in the presence or absence of SB203580. Both DNA fragmentation (detected at the indicated times by diphenylamine method) and phosphatidylserine surface expression (detected by PE-conjugated annexin-V staining and fluorescence-activated cell sorting analysis) were induced by anti-NGF Abs and inhibited by SB203580. Results from one experiment out of three performed are shown.

iting their functions. Moreover, Bcl-2 and Bcl-X_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, 38, 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_L and Bcl-2, respectively, as suggested by the recent demonstration that JNK specifically phosphorylates Thr-47 and Thr-115 residues in Bcl-X_L protein (51); if so, since memory B cells do not express Bcl-X_L (58), the absence of the natural mitochondrial target of JNK would render dispensable the specific cytosolic phosphatase instead needed in PC12 cells that express both Bcl-2 and Bcl-X_L. 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,

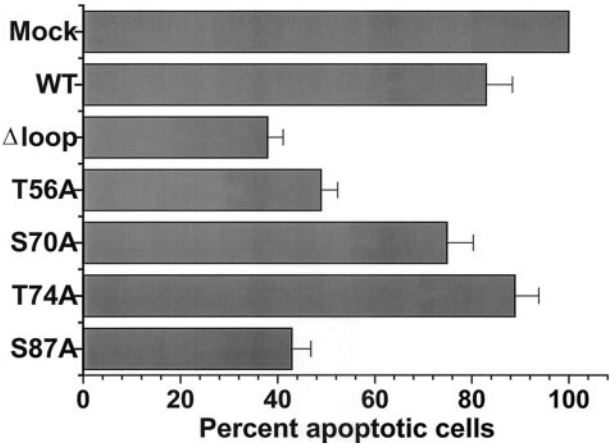

FIG. 6. **Deletion of Bcl-2 loop region or mutation of selected residues within it protects cells from the apoptosis induced by NGF withdrawal.** CESS cells were transiently transfected with constructs carrying Bcl-2 WT or the indicated mutations within a GFP-expressing vector. Transfected cultures were exposed in triplicate to 10 μg/ml anti-NGF Abs or rat IgG as control for 12 h. GFP^+ cells undergoing apoptosis were detected by PE-conjugated annexin-V staining and cytofluorimetric analysis. The percentage of apoptotic cells was defined as the percentage of anti-NGF Abs-treated cells minus that of rat IgG-treated cells. Data were collected as percentage of apoptotic cells shown by each transfectant, relative to mock-transfected cells. Untransfected CESS cells had levels of apoptosis comparable with that of mock transfected cells. Shown are the mean ± S.D. percentage of apoptotic cells from five different experiments. Superimposable results were obtained when K252a was used as apoptotic stimulus.

JNK failed to phosphorylate recombinant Bcl-2, and the addition of activated JNK to mitochondria loaded with Bcl-2 did not elicit cytochrome *c* release, while both phosphorylation of Bcl-2 and the subsequent cell death were blocked by the p38 MAPK inhibitor we used. Although the SB compound was shown to affect also the RAF-1 kinase, paradoxically in a stimulant fashion, leaving the possibility that in our system other enzymes may be involved (64), the latter data, taken together with the above considerations, strongly indicate that in memory B cells the axis NGF receptor-p38 MAPK-Bcl-2 is the major regulator of the death/survival divide.

At present, the phosphatase(s) deactivating p38 MAPK upon NGF receptor signaling is unknown. Instead, recent work has provided hints to understand the trigger initiating the MAPK-activating pathway that leads to p38 MAPK phosphorylation in the absence of NGF. The notion that the $p75^{NTR}$ receptor chain can induce cell death in the absence of its ligands (59) and the demonstration that neurons of transgenic mice expressing a truncated form of $p75^{NTR}$ undergo accelerated apoptosis (60) had pointed to that molecule as the initiator of the process, consistent with our observation that neutralization of either NGF receptor chain causes memory B cell death.[3] Recently, it has been shown that the intracellular portion of $p75^{NTR}$ can bind adaptor molecules, such as TRAF2, when the molecule is in monomeric form (*i.e.* in the absence of extracellular NGF), whereas it binds TRAF6 when dimerized via the extracellular bridging of NGF (61, 62). While oligomerized TRAF6 leads to the nuclear translocation of NF-κB (62), TRAF2 is able to trigger the MAPK pathway down to p38 MAPK activation (63). On the contrary, it is conceivable that, when the ligand-binding extracellular region is occupied by NGF, the intracellular portion of $p75^{NTR}$ binds, directly or indirectly, a p38 MAPK-specific phosphatase (possibly activated upon phosphorylation by Trk), a hypothesis currently being tested in our laboratory.

Our studies were performed essentially on freshly isolated, 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, by 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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[3] M. Torcia, G. De Chiara, and P. Rosini, unpublished results.

REFERENCES

1. Scaffidi, C., Kirchhoff, S., Krammer, P. H., and Peter, M. E. (1999) *Curr. Opin. Immunol.* **11,** 277–285
2. Jacobson, M. D., Weil, M., and Raff, M. C. (1997) *Cell* **88,** 347–354
3. Green, D. R. (1998) *Cell* **94,** 695–698
4. Reed, J. C. (1997) *Nature* **387,** 773–776
5. Adams, J. M., and Cory, S. (1998) *Science* **281,** 1322–1326
6. Green, D. R. (2000) *Cell* **102,** 1–4
7. Brenner, C., Cadiou, H., Vieira, H. L., Zamzami, N., Marzo, I., Xie, Z., Leber, B., Andrews, D., Duclohier, H., Reed, J. C., and Kroemer, G. (2000) *Oncogene* **19,** 329–336
8. Shimizu, S., Konishi, A., Kodama, T., and Tsujimoto, Y. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3100–3105
9. Shimizu, S., Narita, M., and Tsujimoto, Y. (1999) *Nature* **399,** 483–487
10. Vander Heiden, M. G., Li, X. X., Gottleib, E., Hill, R. B., Thompson, C. B., and Colombini, M. (2001) *J. Biol. Chem.* **276,** 19414–19419
11. Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. (1997) *Science* **278,** 1966–1968
12. Haldar, S., Chintapalli, J., and Croce, C. M. (1996) *Cancer Res.* **56,** 1253–1255
13. Haldar, S., Jena, N., and Croce, C. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4507–4511
14. Srivastava, R. K., Mi, Q. S., Hardwick, J. M., and Longo, D. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3775–3780
15. Navarro, P., Valverde, A. M., Benito, M., and Lorenzo, M. (1999) *J. Biol. Chem.* **274,** 18857–18863
16. Ojala, P. M., Yamamoto, K., Castanos-Velez, E., Biberfeld, P., Korsmeyer, S. J., and Makela, T. P. (2000) *Nat. Cell Biol.* **2,** 819–825
17. Rosini, P., De Chiara, G., Lucibello, M., Garaci, E., Cozzolino, F., and Torcia, M. (2000) *Biochem. Biophys. Res. Commun.* **278,** 753–759
18. Ito, T., Deng, X., Carr, B., and May, W. S. (1997) *J. Biol. Chem.* **272,** 11671–11673
19. Ruvolo, P. P., Deng, X., Carr, B. K., and May, W. S. (1998) *J. Biol. Chem.* **273,** 25436–25442
20. Ruvolo, P. P., Deng, X., Ito, T., Carr, B. K., and May, W. S. (1999) *J. Biol. Chem.* **274,** 20296–20300
21. Dimmeler, S., Breitschopf, K., Haendeler, J., and Zeiher, A. M. (1999) *J. Exp. Med.* **189,** 1815–1822
22. Breitschopf, K., Haendeler, J., Malchow, P., Zeiher, A. M., and Dimmeler, S. (2000) *Mol. Cell. Biol.* **20,** 1886–1896
23. Levi-Montalcini, R. (1987) *Science* **237,** 1154–1162
24. Torcia, M., Bracci-Laudiero, L., Lucibello, M., Nencioni, L., Labardi, D., Rubartelli, A., Cozzolino, F., Aloe, L., and Garaci, E. (1996) *Cell* **85,** 345–356
25. Greenlund, L. J., Korsmeyer, S. J., and Johnson, E. M., Jr. (1995) *Neuron* **15,** 649–661
26. Katoh, S., Mitsui, Y., Kitani, K., and Suzuki, T. (1996) *Biochem. Biophys. Res. Commun.* **229,** 653–657
27. Pincelli, C., Haake, A. R., Benassi, L., Grassilli, E., Magnoni, C., Ottani, D., Polakowska, R., Franceschi, C., and Giannetti, A. (1997) *J. Invest. Dermatol.* **109,** 757–764
28. Kummer, J. L., Rao, P. K., and Heidenreich, K. A. (1997) *J. Biol. Chem.* **272,** 20490–20494
29. Maroney, A. C., Finn, J. P., Bozyczko-Coyne, D., O'Kane, T. M., Neff, N. T., Tolkovsky, A. M., Park, D. S., Yan, C. Y., Troy, C. M., and Greene, L. A. (1999) *J. Neurochem.* **73,** 1901–1912
30. Le Niculescu, H., Bonfoco, E., Kasuya, Y., Claret, F. X., Green, D. R., and Karin, M. (1999) *Mol. Cell. Biol.* **19,** 751–763
31. Horstmann, S., Kahle, P. J., and Borasio, G. D. (1998) *J. Neurosci. Res.* **52,** 483–490
32. Chu, Y., Solski, P. A., Khosravi-Far, R., Der, C. J., and Kelly, K. (1996) *J. Biol. Chem.* **271,** 6497–6501
33. Tibbles, L. A., and Woodgett, J. R. (1999) *Cell Mol. Life Sci.* **55,** 1230–1254
34. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. (1996) *Mol. Cell. Biol.* **16,** 1247–1255
35. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) *Cell* **87,** 619–628
36. Wang, H. G., Rapp, U. R., and Reed, J. C. (1996) *Cell* **87,** 629–638
37. Malmqvist, M., and Karlsson, R. (1997) *Curr. Opin. Chem. Biol.* **1,** 378–383
38. Chang, B. S., Minn, A. J., Muchmore, S. W., Fesik, S. W., and Thompson, C. B. (1997) *EMBO J.* **16,** 968–977
39. Lee, J. C., Kassis, S., Kumar, S., Badger, A., and Adams, J. L. (1999) *Pharmacol. Ther.* **82,** 389–397

40. Maundrell, K., Antonsson, B., Magnenat, E., Camps, M., Muda, M., Chabert, C., Gillieron, C., Boschert, U., Vial-Knecht, E., Martinou, J. C., and Arkinstall, S. (1997) *J. Biol. Chem.* **272,** 25238–25242
41. Yamamoto, K., Ichijo, H., and Korsmeyer, S. J. (1999) *Mol. Cell. Biol.* **19,** 8469–8478
42. Basu, A., You, S. A., and Haldar, S. (2000) *Int. J. Oncol.* **16,** 497–500
43. Fan, M., Goodwin, M., Vu, T., Brantley-Finley, C., Gaarde, W. A., and Chambers, T. C. (2000) *J. Biol. Chem.* **275,** 29980–29985
44. Thomas, A., Giesler, T., and White, E. (2000) *Oncogene* **19,** 5259–5269
45. Johnson, L. N., and Barford, D. (1993) *Annu. Rev. Biophys. Biomol. Struct.* **22,** 199–232
46. Fang, X., Yu, S., Eder, A., Mao, M., Bast, R. C., Boyd, D., and Mills, G. B. (1999) *Oncogene* **18,** 6635–6640
47. Verma, S., Zhao, L., and Chinnadurai, G. (2001) *J. Biol. Chem.* **276,** 4671–4676
48. Zhu, W., Cowie, A., Wasfy, G. W., Penn, L. Z., Leber, B., and Andrews, D. W. (1996) *EMBO J.* **15,** 4130–4141
49. Green, D. R., and Reed, J. C. (1998) *Science* **281,** 1309–1312
50. Melamed, I., Turner, C. E., Aktories, K., Kaplan, D. R., and Gelfand, E. W. (1995) *J. Exp. Med.* **181,** 1071–1079
51. Kharbanda, S., Saxena, S., Yoshida, K., Pandey, P., Kaneki, M., Wang, Q., Cheng, K., Chen, Y. N., Campbell, A., Sudha, T., Yuan, Z. M., Narula, J., Weichselbaum, R., Nalin, C., and Kufe, D. (2000) *J. Biol. Chem.* **275,** 322–327
52. Tamura, K., Sudo, T., Senftleben, U., Dadak, A. M., Johnson, R., and Karin, M. (2000) *Cell* **102,** 221–231
53. Kroemer, G., and Reed, J. C. (2000) *Nat. Med.* **6,** 513–519
54. Kirsch, D. G., Doseff, A., Chau, B. N., Lim, D. S., Souza-Pinto, N. C., Hansford, R., Kastan, M. B., Lazebnik, Y. A., and Hardwick, J. M. (1999) *J. Biol. Chem.* **274,** 21155–21161
55. Clem, R. J., Cheng, E. H., Karp, C. L., Kirsch, D. G., Ueno, K., Takahashi, A., Kastan, M. B., Griffin, D. E., Earnshaw, W. C., Veliuona, M. A., and Hardwick, J. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 554–559
56. Chadebech, P., Brichese, L., Baldin, V., Vidal, S., and Valette, A. (1999) *Biochem. Biophys. Res. Commun.* **262,** 823–827
57. Wang, S., Wang, Z., Boise, L., Dent, P., and Grant, S. (1999) *Biochem. Biophys. Res. Commun.* **259,** 67–72
58. Grillot, D. A., Merino, R., Pena, J. C., Fanslow, W. C., Finkelman, F. D., Thompson, C. B., and Nunez, G. (1996) *J. Exp. Med.* **183,** 381–391
59. Bredesen, D. E., and Rabizadeh, S. (1997) *Trends Neurosci.* **20,** 287–290
60. Majdan, M., Lachance, C., Gloster, A., Aloyz, R., Zeindler, C., Bamji, S., Bhakar, A., Belliveau, D., Fawcett, J., Miller, F. D., and Barker, P. A. (1997) *J. Neurosci.* **17,** 6988–6998
61. Ye, X., Mehlen, P., Rabizadeh, S., VanArsdale, T., Zhang, H., Shin, H., Wang, J. J., Leo, E., Zapata, J., Hauser, C. A., Reed, J. C., and Bredesen, D. E. (1999) *J. Biol. Chem.* **274,** 30202–30208
62. Khursigara, G., Orlinick, J. R., and Chao, M. V. (1999) *J. Biol. Chem.* **274,** 2597–2600
63. Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y., and Karin, M. (1999) *Genes Dev.* **13,** 1297–1308
64. Hall-Jackson, C. A., Goedert, M., Hedge, P., and Cohen, P. (1999) *Oncogene* **18,** 2047–2054