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NGF Withdrawal Induces Apoptosis in CESS B Cell Line through p38 MAPK Activation and Bcl-2 Phosphorylation

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The sIgG$^+$ lymphoblastoid B cell line CESS spontaneously produces a high amount of NGF and expresses both high affinity (p140$^{Trk\text{-}A}$) and low affinity (p75^{NTR}) NGF receptors. Blocking NGF signals with neutralizing antibodies or specific Trk-A inhibitors induces a rapid phosphorylation of antiapoptotic Bcl-2 protein, followed by caspase activation, and apoptotic death of CESS cells. Bcl-2 phosphorylation in several sites within a ≈60 aa "loop" domain of protein is known to regulate its antiapoptotic function. Accordingly, CESS cells expressing the loop deletional mutant cDNA constructs Bcl-2 Δ40-91 were completely resistant to apoptosis induced by NGF withdrawal, indicating that Bcl-2 phosphorylation is a critical event. NGF withdrawal induces p38 MAPK, but not JNK, activation in CESS cells, and SB203580, a specific inhibitor of p38 MAPK, is able to prevent both Bcl-2 phosphorylation and apoptosis, indicating that p38 MAPK is the enzyme responsible for these events.

Key Words: **apoptosis; nerve growth factor; p38 MAPK; lymphoblastoid cells; Bcl-2; phosphorylation.**

Apoptosis plays an indispensable role in the development and maintenance of homeostasis within all multicellular organisms. In the immune system, apoptosis contributes to limit the clonal expansion of lymphocytes during an immune response against a foreign antigen. Once the antigen has been successfully eliminated, a precipitous fall in lymphocyte numbers as well as a diminution in effector functions occur, which are related to the gradual wane of the stimuli needed for lymphocyte survival, including costimulators and cytokines. The biochemical hallmark of these phenomena is the reduced expression of the antiapoptotic Bcl-2 family proteins (1).

The antiapoptotic function of Bcl-2 is mainly exerted at the mitochondrial site through the formation of well-balanced molecular complexes with other proapoptotic (Bax) or antiapoptotic (Bcl-X_L) members of the family, which control the efflux of cytochrome *c* and other mitochondrial components able to activate the caspase cascade (2–4). A direct binding with mitochondrial proteins, such as adenine nucleotide traslocator (ANT) and the voltage-dependent anion channel (VDAC), has also been suggested as part of the regulatory functions of Bcl-2 voted to prevent cytochrome *c* release (5, 6). The delicate nature of these protein-protein interactions explains why factors affecting Bcl-2 protein expression and/or function are able to modulate apoptotic cell death. In this context, phosphorylation of Bcl-2 residues within the so called "loop" region between α1 and α2 helices has been considered a major regulatory event (7–12).

Nerve Growth Factor (NGF) is a neurotrophic polypeptide that is necessary for survival of various neuronal cell populations (13), whereby NGF deprivation induces Bcl-2 down-regulation and neuronal apoptosis (14). Within the immune system, memory B cells spontaneously produce Nerve Growth Factor (NGF) and express high and low affinity NGF receptors, creating an autocrine circuit which provides B cells with the ability to survive through the maintenance of Bcl-2 protein integrity (15). Albeit it was discovered decades ago, a clear definition of the molecular events linking its binding to the surface receptors with modulation of those pathways that affect Bcl-2 protein still is lacking. Here, we describe that the lymphoblastoid cell line CESS expresses functional properties similar to those of memory B lymphocytes, as it produces and utilizes NGF as an autocrine survival factor. In CESS cells, apoptosis induced by NGF deprivation is related to Bcl-2 phosphorylation, since Bcl-2 mutants lacking the target amino acids are resistant to apoptosis. We also show that p38 MAPK is the enzyme responsible for Bcl-2 phosphorylation under these experimental conditions.

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MATERIALS AND METHODS

Reagents. Neutralizing rat anti-human NGF mABs (clone αD11) was kindly donated by Dr. A. Cattaneo (SISSA, Trieste), and always used at 10 μg/ml. Rabbit anti-human JNK, anti-human phosphorylated JNK, anti-human actin, goat and mouse anti-human Bcl-2 Abs, anti-human PARP were purchased by Santa Cruz Biotechnology. Rabbit anti-human p38 MAPK and anti-human phosphorylated p38 MAPK was purchased by New England Biolabs. Mouse anti-human p75NTR and anti-human gp140 Trk-A monoclonal antibodies were a generous gift of Dr. S. Alemà (Institute of Neurobiology, CNR, Rome) and hrNGF was a generous gift of Dr. G. Ferrari (Fidia, Abano, Italy). The p38 MAPK inhibitor SB203580, the inactive SB202474, the Trk-A inhibitors Tyrphostin AG879, the inactive molecule Tyrphostin A1 and K252a were purchased by Calbiochem.

CESS cell line was purchased by ATCC and cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine.

Phenotypic analysis. CESS cells were stained with FITC anti-human IgG, anti-human IgD polyclonal antibodies or with anti-CD20, anti-CD19, anti-CD38, anti-CD44 (Alexis Corp.), anti-CD77 (Coulter, Fisher Scientific Corp.), monoclonal antibodies followed by FITC anti-mouse IgG (NEN) and analyzed by cytofluorimeter. p75NTR and gp140 Trk-A and NGF expression were studied by Western blot analysis with specific antibodies.

ELISA. NGF production was also assessed by ELISA as described by Söderström *et al.* (16) by using a commercially available NGF mouse monoclonal antibody (mAb) 27/21 (Boehringer Mannheim, Mannheim, Germany). The sensitivity of the test was ranging between 8 and 20 pg/ml.

Proliferation assay. CESS cells were cultured in 96-well plates at 5×10^4/ml in RPMI 1640 medium supplemented with 5% FCS for 18 h. 0.5 μCi of ^{3}H-Thymidine were added in each well during the last 8 h of culture. Cells were harvested and radioactivity recorded in a β-counter.

Apoptosis assay. CESS cells were cultured for different times at 5×10^6/ml, with 10 μg/ml anti-NGF mAb or control rat IgG, with 50 μM tyrphostin AG879 or Tyrphostin A1, with 100 nM K252a in RPMI medium in the presence or absence of 25 μM SB203580 or SB202474. Cells 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 analysis of PARP cleavage, cells, cultured for 6 h as above, were lysed and immunoblotted with specific anti-human PARP antibodies.

Phosphorylation assays. To assess phosphorylation of p38 MAPK, JNK or Bcl-2 protein, 5×10^6 CESS cells (nontransfected or after 3 days of transfection) were cultured as indicated, then lysed in a buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, Phosphatase Inhibitors Cocktails (Sigma), 1 mM PMSF, 1 μg/ml pepstatin A, 5 μg/ml aprotinin, 5 μg/ml leupeptin; equal amounts (40 μg) of lysates were run in 15% SDS/PAGE, blotted onto nitrocellulose filters, and stained with anti-human phosphorylated p38 MAPK, anti-human phosphorylated JNK or goat anti-Bcl-2 IgG preparation known to recognize antigenic determinants also outside the loop region.

Construction of Bcl-2 mutants. The full-length human-*bcl-2* sequence was amplified by PCR 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: forward, 5′-TTAGATCTATGGCGCACGCTGGGAGAAC-3′; reverse, 5′-CCGAATTCTCACTTGTGGCTCAGATAGG-3′, yielding the WT construct. The Δ-loop plasmid, containing the *bcl-2* deletion mutant (Δ 40-91 aa), was generated by two-step recombinant PCR using WT plasmid as template and two oligonucleotide pairs: 5′-CCACGGGCCCGGCGCCCACATCTCCCGC-3′ with 5′-GCAGAGCTGGTTTAGTGAACCGTCA-3′ and 5′-GGGCCCGTGGTCCACCTGGCCCTCCGCCAA-3′ with 5′-TATTCCAAGCGGCTTCGGCCAGTAA-3′.

The two PCR products were mixed in the same PCR 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 and used after sequencing analysis.

Cell culture, transient transfections, and cytofluorimetric analysis. Transient transfections of CESS cells were obtained by Lipofectamine (Gibco BRL). 2×10^6 cells were seeded into 6-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 $\geq$30%. Apoptotic stimuli (10 μg/ml of anti-NGF Abs, or 50 μM Tyrphostyn AG879 or 100 nM K252a for 12 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

Expression of NGF and NGF Receptors by CESS Cells

We had observed previously that memory B lymphocytes express low and high affinity receptors for NGF and produce large amounts of the cytokine (15). To find out an *in vitro* established model of memory B cells that could be used to extend our studies, we screened several lymphoblastoid cell lines for the above parameters, focussing on those expressing the IgD^- surface phenotype, typical of a late B cell differentiation stage. Amongst these, we selected the CESS cell line, which is an EBV-transformed $EBNA^+$ cell line, that displays a $CD19^+$, $CD20^-$, $CD44^+$ $CD38^+$, $CD77^-$, $IgGk^+$ surface phenotype, as assessed by a cytofluorimetric analysis performed with a panel of monoclonal antibodies. The class of membrane Ig receptors is consistent with the hypothesis that the normal cell originating this cell line was an antigen-selected, somatically hypermutated, proliferating B lymphocyte, a stage ontogenetically close to that of memory B cell (17).

Immunoblot analysis of lysates and supernatants with specific anti-NGF antibodies showed that CESS cells spontaneously produce and release NGF in culture supernatants (Fig. 1A). These data were confirmed by ELISA measurement of NGF which revealed a production of 180 ± 13 pg/10^6 cells during 12 h of incubation. Then, CESS cells were studied for NGF receptor expression by immunoblot analysis with anti-human $p75^{NTR}$ and anti-human $gp140^{Trk\text{-}A}$ antibodies. Figures 1B and 1C show that CESS cells express both low and high affinity NGF receptors. On the whole, the above data confirm that CESS cells and memory B cells have a superimposable pattern of NGF and NGF receptor expression, suggesting the existence of an autocrine NGF circuit.

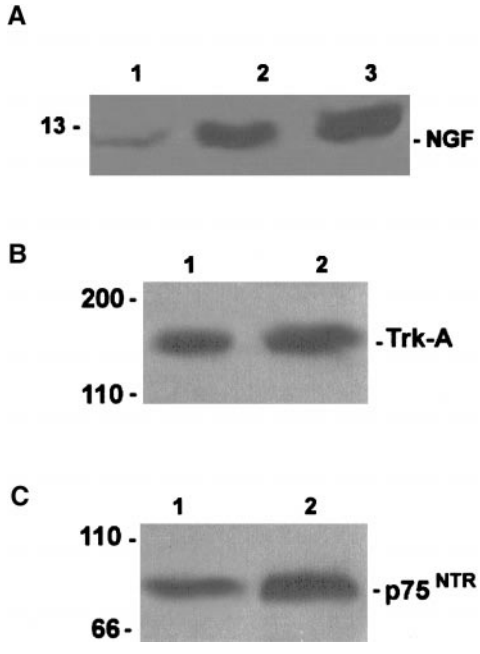


FIG. 1. CESS cells produce NGF and express NGF receptors. (A) Immunoblot analysis of lysates (lane 1) and supernatants (lane 2) from CESS cells with anti-NGF antibodies; lane 3, recombinant NGF. (B–C) Immunoblot analysis of lysates from CESS cells (lane 1) or PC12 cells (lane 2) with anti-Trk-A or anti-$p75^{NTR}$ antibodies.

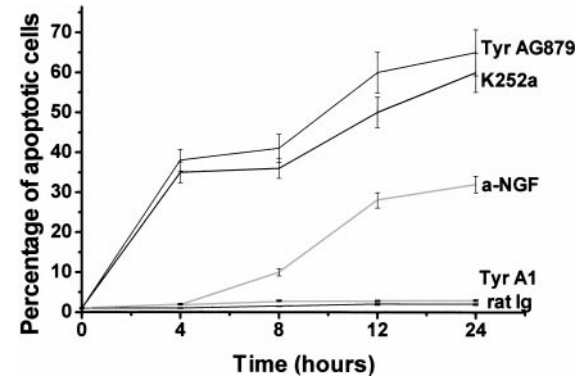


FIG. 2. NGF withdrawal induces apoptotic cell death. CESS cells were incubated for the indicated times with 10 μg/ml anti-NGF antibodies or control IgG, with 50 μM tyrphostin AG879 or tyrphostin A1, or with 100 nM K252a, washed, stained with PE-conjugated annexin-V. Percentage of apoptotic cells was obtained by cytofluorimetric analysis.

NGF Withdrawal Induces Apoptotic Cell Death

To investigate the functional role of endogenous NGF, cell proliferation experiments were performed in the presence or absence of exogenous recombinant NGF, of neutralizing antibodies to NGF, or specific Trk-A inhibitors, such as K252a and Tyrphostin AG879 (18, 19). Table 1 shows that, while exogenous NGF induced only a minimal cell proliferation, the addition of anti-NGF antibodies or Trk-A inhibitors caused a marked reduction of spontaneous thymidine incorporation after 18 h of culture; cell viability, assessed by Trypan blue dye exclusion, yielded comparable results (not shown).

TABLE 1

Effect of NGF and NGF Neutralizing Agents on ^{3}H-Thymidine Incorporation by CESS Cells

^{3}H-Thymidine incorporation in the presence of	
Medium	5354 ± 563
NGF (100 ng/ml)	8745 ± 643
NGF (50 ng/ml)	7326 ± 754
NGF (25 ng/ml)	5975 ± 437
a-NGF (10 μg/ml)	2437 ± 197
Rat IgG (10 μg/ml)	5845 ± 598
Tyrphostin AG879 (50 μM)	853 ± 165
Tyrphostin A1 (50 μM)	5875 ± 236
K252a (200 nM)	923 ± 152

Note. CESS cells were cultured at 5×10^4/ml in RPMI 1640 medium with 5% FCS for 18 h. Results are expressed as mean ^{3}H-Thymidine incorporation ± SD of triplicate cultures. Data from one representative experiment out of three performed are shown.

To assess whether NGF neutralization induced apoptotic cell death, CESS cells, cultured at different time intervals with or without K252a, or Tyrphostin AG879 or antibodies to NGF, were stained with PE-annexin-V to detect exposure of membrane phosphatidylserine, as a marker of apoptosis. Figure 2 shows that all the above reagents induced apoptotic cell death, with different efficiency and timing, as the Trk-A inhibitors were able to induce apoptosis after 4 h of incubation, while anti-NGF antibodies induced the maximum effect, which was of lower intensity, after 12 h of incubation. These findings confirm that CESS cells utilize NGF as an autocrine survival factor, and are consistent with the analysis of PARP cleavage that revealed activation of caspase 3-like molecule (see below).

NGF Withdrawal Induces Bcl-2 Phosphorylation and 40–91 Δ-Loop Bcl-2 Construct Protect CESS Cells from Apoptosis

The rapid onset of apoptosis induced by Trk-A inhibitors in CESS cells prompted us to investigate which biochemical pathway was activated by these reagents. First, we wanted to assess expression of the antiapoptotic Bcl-2 and Bcl-X_L proteins upon NGF withdrawal. Figure 3 shows that, after 12 h of incubation, all the NGF neutralizing reagents induced a marked reduction of Bcl-2 protein levels. Bcl-X_L expression by CESS cells could never be detected (not shown).

Since the apoptotic process induced by Trk-A inhibitors had a much more rapid kinetics, as revealed by annexin-V staining and PARP cleavage, we looked for a more proximal metabolic event that could trigger apop-

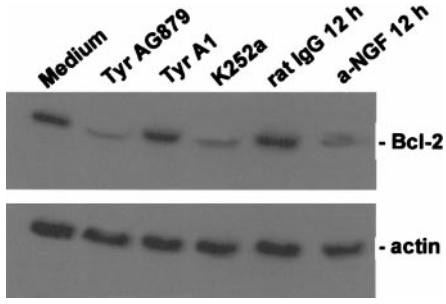


FIG. 3. Bcl-2 expression is strongly reduced after NGF withdrawal. CESS cells were incubated for 12 h with anti-NGF antibodies, control IgG, tyrphostin AG879, tyrphostin A1, or K252a, lysed and immunoblotted with anti-Bcl-2 antibodies, then with anti-actin antibodies as protein loading control.

tosis. In fact, phosphorylation of Bcl-2 proteins has been reported by several authors as a posttranslational modification able to affect the antiapoptotic function of the protein, either positively or negatively (7–12). We therefore studied whether Bcl-2 phosphorylation status was related to the onset of apoptosis induced by NGF deprivation, by culturing CESS cells with NGF neutralizing agents for a time interval shorter than that needed to induce apoptosis. Figure 4 shows a clear mobility shift of Bcl-2 protein, from the native 26-kD to the phosphorylated 29-kD form after only 2 h of incubation with Tyrphostin AG879 or K252a, or after 6 h of incubation with anti-NGF antibodies.

To further investigate whether Bcl-2 phosphorylation could be related to the onset of apoptosis, we cloned Bcl-2 wild type (WT) cDNA and a mutant Bcl-2 cDNA bearing a deletion of the entire loop (Δ-loop) region in plasmids coding for green fluorescent protein (GFP) as reporter. CESS cells were transiently transfected with the above constructs or with a void plasmid as control; then, after 3 days transfected cultures were treated with anti-NGF antibodies or with Tyrphostin AG879 or K252a for 12 h, and apoptosis of transfected GFP^+ cells recorded by staining with PE-annexin V in a cytofluorimetric analysis. Figure 5A shows that, although a certain level of protection from apoptosis was achieved in cells transfected with WT plasmid, as compared to native CESS cells or to mock-transfected cells, the Δ-loop mutant conferred strong resistance to apoptosis in all of the conditions tested. Immunoblot analysis of lysates from transfected cultures showed that, while endogenous or transfected 26-kD Bcl-2 protein underwent phosphorylation (as revealed by mobility shift bands), the 19-kD Δ-loop Bcl-2 protein did not (Fig. 5B).

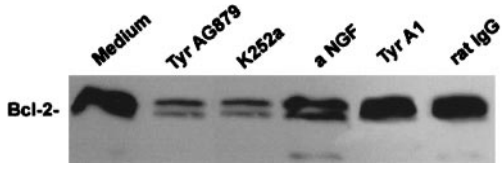
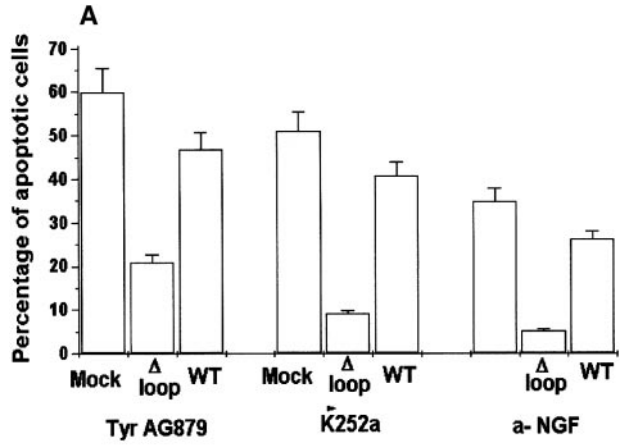


FIG. 4. NGF withdrawal induces Bcl-2 phosphorylation. CESS cells were incubated for 2 h with K252a, tyrphostin AG879, or tyrphostin A1, and for 6 h with anti-NGF antibodies or control IgG. Cells were lysed in the appropriate buffer, run on 15% SDS–PAGE and immunoblotted with anti-Bcl-2 antibodies. Phosphorylation is evident as a band mobility shift.



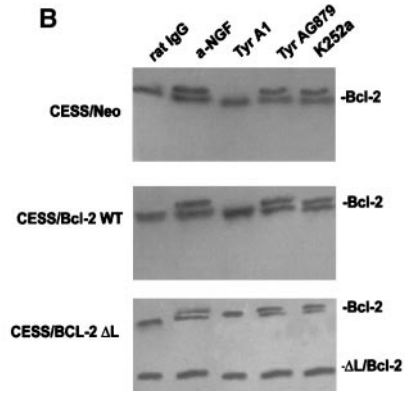


FIG. 5. Expression of Δ-loop Bcl-2 protein protects CESS cells from apoptosis induced by NGF withdrawal. CESS cells were transiently transfected with constructs carrying WT Bcl-2, Δ-loop Bcl-2, or a void plasmid within a GFP-expressing vector. Transfected cultures were exposed in triplicate to anti-NGF antibodies, K252a, or tyrphostin AG879. GFP^+ cells undergoing apoptosis were detected by PE-conjugated annexin-V staining and cytofluorimetric analysis. When exposed to control rat IgG, tyrphostin A1, or medium alone, ≈3% of transfected cells underwent apoptosis.

These data indicate that preventing Bcl-2 serine or threonine phosphorylation, through the expression of a Δ-loop Bcl-2 mutant, protects CESS cells from apoptosis induced by NGF withdrawal.

p38 MAPK Is Activated upon NGF Withdrawal and Is Responsible for Bcl-2 Phosphorylation

In order to identify the Bcl-2 phosphorylating enzymes, we mainly focused on two enzymes of the

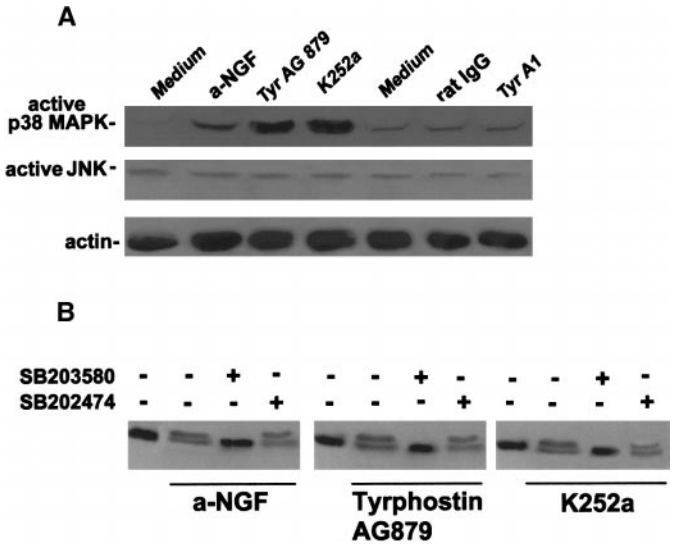


FIG. 6. p38 MAPK, but not JNK, is activated by NGF withdrawal in CESS cells and involved in Bcl-2 phosphorylation. (A) CESS cells were incubated for 1 h with the indicated stimuli, lysed and immunoblotted with antibodies to phosphorylated p38 MAPK or phosphorylated JNK, and to actin as protein loading control. (B) CESS cells were incubated with anti-NGF antibodies for 6 h, with tyrphostin AG879, with K252a for 2 h, in the presence or absence of 25 μM SB203580 or of the inactive SB202474. Cells were lysed with the appropriate buffer and stained with anti-Bcl-2 antibodies. SB203580 inhibits Bcl-2 phosphorylation in all the conditions tested.

mitogen-activated protein kinase (MAPK) superfamily, p38 MAPK and JNK, since these enzymes were reported to be activated upon NGF or other growth factor deprivation in different systems and to be involved in the initiation of the apoptotic process (20, 21). In particular, JNK was specifically involved as the enzyme responsible for Bcl-2 phosphorylation induced by taxanes and DNA-damaging drugs (vinblastin) (8, 22–24).

CESS cells were cultured at different time intervals with anti-NGF antibodies, Tyrphostin AG879, or K252a, and phosphorylation, as the marker of enzyme activation, assessed. Figure 6A shows that p38 MAPK was strongly activated after 1 h of treatment (and its phosphorylation persisted up to 12 h [not shown]), while JNK was never activated under the same experimental conditions. Consistently, the specific p38 MAPK inhibitor SB203580 (25) was able to block Bcl-2 phosphorylation induced by NGF withdrawal in CESS cells (Fig. 6B), while the SB202474 compound, used as control molecule, was inactive.

Next, to confirm the prominent role of p38 MAPK activation in the apoptotic process induced by NGF deprivation, we cultured CESS cells with anti-NGF antibodies, with Tyrphostin AG879, or K252a for 12 h, in the presence or absence of the specific p38 MAPK inhibitor SB203580 or the inactive molecule SB202474 as control, and apoptosis was assessed by annexin-V staining and cytofluorimetric analysis. Activation of caspase 3-like enzymes in these experimental conditions was also assessed by analysis of PARP cleavage. Figures 7A and 7B show that SB203580, but not SB202474, inhibited caspase activation and apoptotic cell death induced by NGF neutralizing agents. These data strongly indicate that p38 MAPK, and not JNK, is responsible for Bcl-2 phosphorylation, and hence apoptotic death, occurring upon NGF deprivation in CESS cells.

DISCUSSION

The CESS cell line is an $sIgG^+$ EBV-transformed lymphoblastoid cell line that express NGF receptors and produce NGF. While both $p75^{NTR}$ and $gp\text{-}140^{Trk\text{-}A}$ NGF receptors are largely expressed by lymphoblastoid cell lines, independently from their maturational stage (26–28), production of NGF is not a common feature of these cells. We had demonstrated that amongst normal B lymphocytes, only the $sIgD^-$ B cell subpopulation, long-living lymphocytes already selected by the antigen, i.e., memory cells, produce the cytokine. Accordingly, Pica *et al.* (28) demonstrated NGF production by BC-1 cells, derived from a primary effusion lymphoma, a cell line expressing numerous

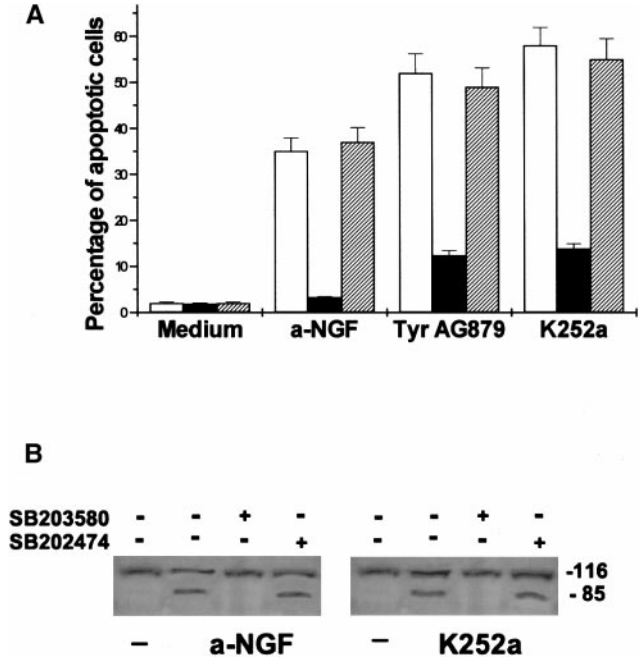


FIG. 7. Inhibition of p38 MAPK prevents caspase activation and apoptosis of CESS cells following NGF withdrawal. (A) CESS cells were incubated for 12 h with anti-NGF antibodies ([web]99[/web]) or for 4 h with Tyrphostin AG879 ([web]99[/web]) or K252a ([web]99[/web]), in the presence or absence of 25 μM SB203580 ([web]103[/web]) or SB202474 ([web]99[/web]). Cells were washed, stained with PE-conjugated annexin-V and analyzed by cytofluorimetry. (B) CESS cells were cultured as above, lysed and immunoblotted with anti-PARP antibodies. Appearance of the 85-kD cleavage fragment indicates activation of caspase 3-like enzymes.

point mutations in the variable region of the Ig heavy chain characteristic of germinal or postgerminal center lymphocytes (29), but not by RAMOS cells that display an $sIgM^+$ phenotype. Although NGF production may be related to viral infection, preliminary data show that NGF production is restricted to lymphoblastoid cell lines with an $sIgD^-$ surface phenotype, suggesting that the feature is related to the maturational stage of B cells. CESS cells utilize NGF as an autocrine survival factor just as the normal, memory B cell, do. In both cell types NGF maintains the expression of Bcl-2 protein, as described in systems other than lymphoid cells (neural cells, keratinocytes (14, 30). In NGF-dependent neuronal cells, factor withdrawal induces down-modulation of the *bcl-2* gene expression and apoptosis (14); in CESS cell line, the quite rapid onset of apoptosis led us to suggest another mechanism regulating Bcl-2 protein levels and function that adds up to down-modulation of gene expression.

Recent studies have demonstrated that Bcl-2 is the target of posttraslational modifications, such as phosphorylation in serine and threonine residues of an unstructured "loop" region between the α1 and α2 helices of the molecule (8, 31). Phosphorylation in these sites has been reported to impair the antiapoptotic effect of Bcl-2 by reducing heterodimer formation with Bax molecules (12), thus affecting the complex molecular structures which regulate mitochondrial cytochrome *c* efflux, able to initiate caspase activation and the apoptosis process. Accordingly, we demonstrate here that Bcl-2 phosphorylation induced by NGF withdrawal is directly linked to apoptosis, since loop deletion mutants of Bcl-2 protein, which cannot undergo phosphorylation, strongly protect CESS cells from apoptosis induced by NGF withdrawal. As a following event, Bcl-2 phosphorylation may render the protein susceptible to proteolytic enzymes, a hypothesis currently under investigation in our laboratory. In this context, it is noteworthy that the loop domain of Bcl-2 was shown to be cleaved by caspases (32)., and that the cleavage product p23-Bcl-2 promotes apoptosis (32, 33).

We also describe the critical role of p38 MAPK activation in the apoptosis induced by NGF withdrawal in CESS cells. These data are in agreement with others (21), who showed p38 MAPK and JNK activation in PC12 cells. However, in the present paper, we report that p38 MAPK is solely involved in Bcl-2 phosphorylation and apoptosis, as demonstrated by several points of evidence, principally by the blocking effects of the p38 MAPK specific inhibitor SB203580. Furthermore, although JNK was described as the enzyme responsible for Bcl-2 phosphorylation induced by taxanes and DNA-damaging drugs (8, 22, 24), we were not able to demonstrate its involvement in apoptosis induced by NGF withdrawal, as its activation was not evident during NGF deprivation. Furthermore, the spectrum of Bcl-2 phosphorylation induced by NGF withdrawal in CESS cells revealed only one band of phosphorylated protein (see Figs. 4–6), while JNK was reported to induce at least three bands of Bcl-2 phosphorylation (23), suggesting that more than one enzyme may phosphorylate Bcl-2, possibly in a diverse combination of residues.

On the whole, our data suggest that NGF deprivation induces apoptosis through activation of p38 MAPK and Bcl-2 phosphorylation. We hypothesize that the role of NGF in factor-dependent cell systems, such as normal memory B cells or neuronal cells, is to prevent kinase activation, probably by maintaining the continuous expression of p38 MAPK-specific phosphatases, that become activated upon interaction with the NGF receptor complex. The availability of a continuous cell line presenting a set of well-defined biochemical events should provide considerable help in the understanding of NGF signaling in normal systems.

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